Symposia Biologica Hungarica 33

ADVANCES IN PROTOZOOLOGICAL RESEARCH

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Akadémiai Kiadó, Budapest

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Symposia Biologica Hungarica 33

Edited by
MAGDOLNA CS. BERECZKY

The volume contains the lectures held at the 1st International Conference of Hungary on Protozoology, and Memorial Session for József Gelei (1885–1952). The conference was jointly organized by the Biological Section of the Hungarian Academy of Sciences and by the Section of Protozoology of the Hungarian Biological Society between 3rd and 6th September 1985 in Budapest at the Hungarian Academy of Sciences.

The 71 papers cover the achievements of protozoologists from 19 countries. Renowned protozoologists have summarized the development of their field from the time of the death of Gelei to the present days. Research results relating to current taxonomy as well as estuarine, river and marine ecology have been presented. The state of the art of our present knowledge on environmental pollution and its effect on the soil is described.

Numerous studies have been carried out on the taxonomy and epidemiology of veterinary parasites as well as on their chemotherapy associated with infections due to protozoa.

In the section on the human aspects papers were delivered on amoebiasis and research achievements in malaria and other diseases caused by parasites.

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MAGDOLNA CS. BERECZKY

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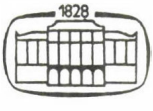
and
Memorial Session for József Gelei (1885–1952),
Budapest, Hungary, September 3–6, 1985

Edited by

MAGDOLNA CS. BERECZKY

Hungarian Danube Research Station,
Hungarian Academy of Sciences,
Göd, Hungary



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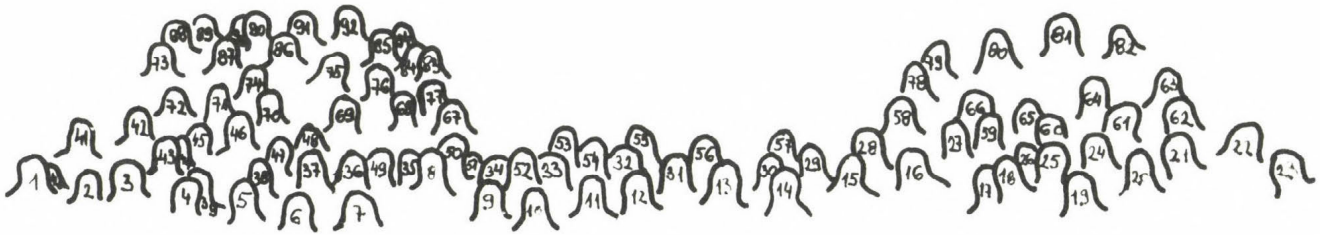
IN MEMORIAM JÓZSEF GELEI (1882-1952)



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Unfortunately, not all participants are present.

PREFACE

I think you will agree with me that, either in Hungary or abroad, there are few protozoologists who are not familiar with the name of József Gelei. He belongs to those scientists who, at the dawn of the rapid development of biology taken in a wider sense, gave directives for a differential development of a branch of biology setting guidelines which have raised the standard of protozoology research.

His talent, steadfast nature, and his observant and logical mind made him pre-eminent among his contemporaries.

In his work he was very exacting, but mainly with himself. He demanded a lot from his coworkers and was outstanding in his research ambitions. He was particularly devoted to his profession, even working when he was seriously ill.

As a man, he was modest but self-respecting. He did not like publicity. However, he was touched by signs of appreciation and affection. Although, as a hot-tempered, impulsive person, he frequently disagreed with his coworkers and students, he always respected them as people. He was a patient examiner. He could not tolerate negligence, inattention and superficiality, but was tolerant with those who did their utmost to achieve success, even if being less talented.

József Gelei is living in our minds as a teacher who set an example in his profession. Now we have assembled to pay homage to the memory of this exceptional man at the centenary of his birth. Grateful thanks are due to all of our kind guests who have honoured us with their presence at this conference.

We dedicate this volume of lectures delivered at the conference to the memory of this great scientist.

The Editor

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WELCOME ADDRESS

Dear Colleagues, Ladies and Gentlemen,

I feel most honoured having the opportunity to address the 1st International Conference of Hungary on Protozoology and Memorial Session for József Gelei on behalf of the Biological Section of the Hungarian Academy of Sciences.

The Hungarian Academy of Sciences was very pleased to be the host of the International Meeting where, besides the Hungarian protozoologists, many famous specialists of this field from all over the world are present. First of all I should like to express our thanks that you all accepted our invitation and so the centenary of the most famous Hungarian protozoologist József Gelei will be commemorated at the highest and mostproper level in the presence of many outstanding protozoologists from 19 countries.

The Biological Section of the Hungarian Academy of Sciences is especially pleased because we are convinced that this meeting will give a positive impetus to the development of Hungarian protozoology which has achieved a very high priority during the last decades not only from the point of view of systematics and classical biology but from that of molecular biology, health and environmental protection.

I should like to express also my personal satisfaction because I was fortunate enough to have known Professor József Gelei, therefore I have a very great admiration of this eminent but, at the same time, reserved and modest personality.

I should like to pay tribute to the members of the Gelei family who were kind enough to join us in commemorating this giant of Hungarian biology.

I declare the 1st International Conference of Hungary on Protozoology and Memorial Session for József Gelei open and wish you all successful discussions and a pleasant time in Budapest.

Professor J. Tigyi

Member of the Hungarian
Academy of Sciences

President of the Biological Section
Hungarian Academy of Sciences

IN MEMORIAM JÓZSEF GELEI
(1882-1952)

Symposia Biologica Hungarica 33 (1986)

SCIENTIFIC CAREER OF J. GELEI

I. TÖRŐ

2nd Department of Anatomy, Semmelweis University Medical School
Budapest, Hungary

In memoriam József Gelei we celebrate a highly active and productive life.

He was born 100 years ago in Árkos in Háromszék county. He came from the same place as Sándor Kőrösi Csoma, János Apáczai and Áron Gábor did, who became internationally well known getting high appreciation for their homeland. Although he was born in a peasant family, his family background bound him to medical sciences. One of his ancestors was Benedek Gelei, who wrote the first medical book in Hungarian in 1600, which has been preserved in manuscript form.

József Gelei completed high school in Kolozsvár which was renowned as a traditional cultural centre of Transylvania over the past centuries. He became a university student there at the Faculty of Exact Sciences in 1903. First he studied natural history and chemistry and later geography. His dream was to be a medical doctor, but financial difficulties forced him to work as a teacher. On encouragement from István Apáthy, he graduated as a biologist and started his scientific career as a demonstrator in Apáthy's Institute in 1905. He soon became an assistant professor and completed his thesis in 1908.

Between 1906 and 1913 he visited Graz, Munich, Würzburg enriching his knowledge with international experience by spending some time in Graff's, Hertwig's and Boweri's Institute.

He was still a student when he started studying the Turbellarians. His monograph on the histology of Turbellarians, published in 1910, was awarded an Academy prize. This was soon

followed by another one for the determination of the ovogenesis of Turbellarians. He described chromosomes in the early leptotene stage in the ovum and he was the first to analyse them in detail.

He discovered a new species of Turbellarians which was accepted by the scientific world as Olistonella Hungarica Gelei. For detailed histological studies on Dendrocoelum lactum, he was awarded the Academy "Vitéz prize" (1912).

He qualified as a 'private docent' in "Comparative studies on cells" in 1914. In 1923 he was only 38 years old when he became a Corresponding Member of the Hungarian Academy of Sciences. A year later in 1924 he became the successor of István Apáthy in the Departments of General Physiology and Comparative Anatomy of the Faculty of Mathematics and Natural Sciences of Szeged University.

The 20 years following his assignment were the most successful period of his life, when he published 124 scientific papers. He established a modern, outstanding institute as illustrated by the photos.

His range of interest was very wide. Besides Turbellarians he studied the structure of nettle cells of limnohydras, water-spraying of coloured shells, and the fauna of lake Balaton. He worked out his new staining method by which he reached his most outstanding results in morphology, physiology and taxonomy of protozoans. He investigated the "neuronema"-systems of the ciliates, the neuroplasmatic fibre network and he clarified the connection between this system and the cilia.

He was the first to analyse their motion.

Gelei stressed the organizational importance of the intercellular spaces in the metazoon against the unicellular organisms and protozoons, stressing intercellular substance, as well as their cytology. He achieved world fame through these investigations. One of the genera of ciliates was named Geleiella in honour of him.

His experimental results were recognized by several invitations abroad and by the Academy membership. He was elected Ordinary Member of the Academy in 1938. His results were ac-

cepted not only in professional studies, but in handbooks and reference books, as well.

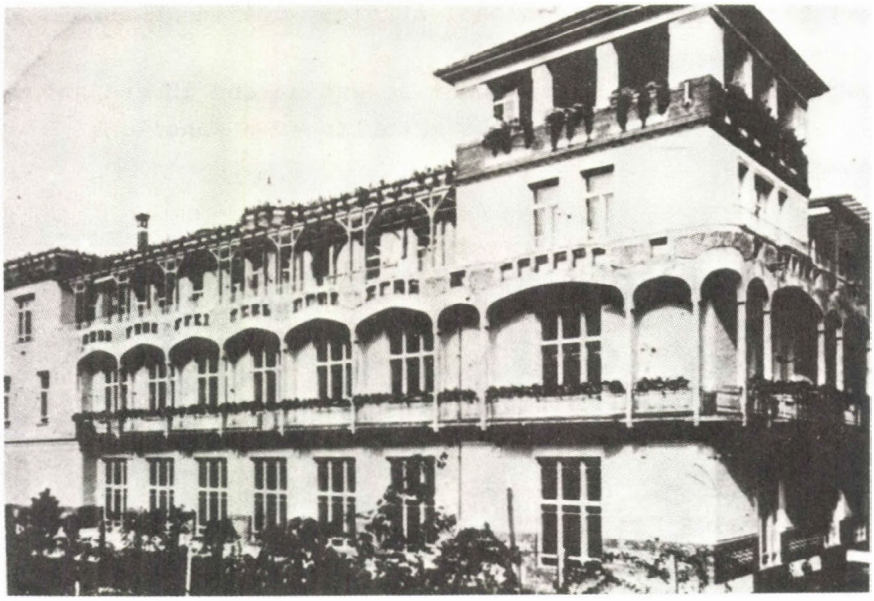
He was not only a prominent scientist and investigator, but an excellent teacher, too, who established a school.

Gelei and his coworkers in Szeged, 1940. Left to right, front row: Stiller, J., Gelei, J., Mátyás, J., Ördög, F., Párducz, B. Back row: Vidacs, J., ?, Horváth, J.

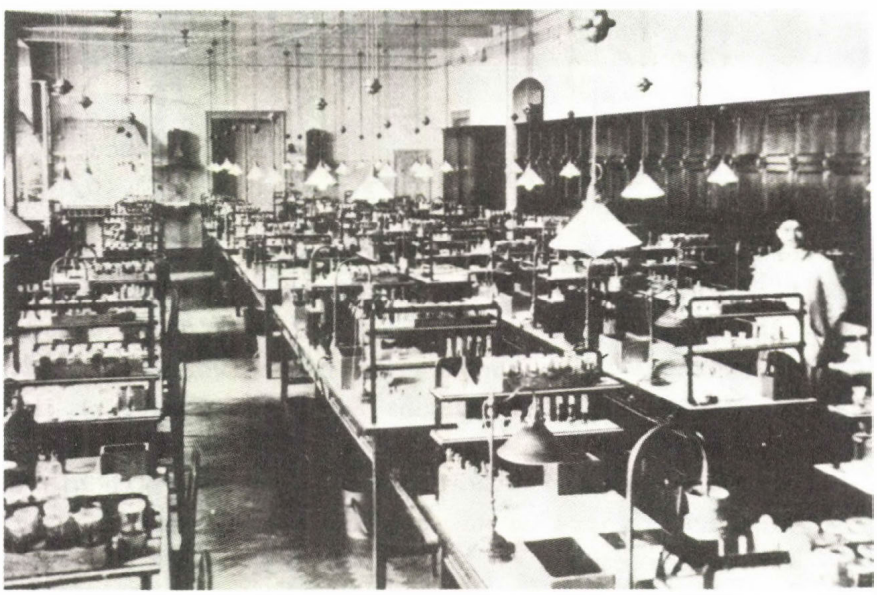
His university-lectures, his activities with undergraduates, his efforts to train postgraduates in experimental work, were all admirable. At the same time, he was charged with the strenuous and responsible work of being the head of the university. He was highly respected through his humane and moral attitudes in the University Council, too.

After the Second World War in 1945, he went with his wife on foot to Szeged, where he was appointed professor of medical biology at the Department of Biology of the Szeged University.

Although his health was already failing at that time, he established a new institute in these difficult years after the war.



Zoological Institute at Kolozsvár



Histology laboratory

An outstanding scientific result of this period of his life was the extension of the basic morphogenetic principle of Severtsov by new ones. He reviewed the pharmacy-training system and introduced general biology into medical education. He was an active teacher when unexpected death carried him away in 1952.

Today, on the 100th anniversary of his birth, when we remember him with honour and recall his particular way of speaking the Transylvanian dialect of Háromszék, his characteristic Székely humour and joviality we are glad to reminisce and celebrate together with his family and students: Jolán Stiller, József Gellért and Dezső Lukács.

The continuity of his activity and his reputation is proved by the fact that even in our days he is one of the most frequently cited Hungarian biologists. We hope that at our Conference, where scientists from all over the world will deliver lectures on several topics, the basic elements established by Gelei will serve as a further proof of the universality of his life-work.

Symposia Biologica Hungarica 33 (1986)

THE PROTOZOOLOGICAL LEGACY OF JÓZSEF GELEI (1885-1952)

M. MÜLLER

Rockefeller University
New York, NY 10021, USA

It is a great honor to me to have been invited to this meeting and it gives me a deep satisfaction to talk about József Gelei whom I admired and regarded an outstanding scientist. His work greatly influenced my own scientific career, if only indirectly. I never had the opportunity to meet him in person, but among those who guided my first steps in science were Jolán Stiller and Béla Párducz, two of Gelei's students and close coworkers (Müller, 1985). Furthermore, my first published paper was a bibliography of his scientific articles which I prepared together with József Gellért, another former student of Gelei (Gellért and Müller, 1954; reprinted: Gellért and Müller, 1986).

We just heard an in-depth analysis of Gelei's signal role in the development of cytology and protozoology in Hungary (Törő, 1986). By his vast theoretical interest, thorough knowledge of a large number of major animal groups in addition to protozoa and mastery of techniques of microscopic investigation he was clearly one of the leading biologists of Hungary of his time. He was also highly successful in attracting students to zoology and trained a significant number of protozoologists, and zoologists in general, who also left their mark on our science. It is relatively easy to assess the significance of an individual scientist for his native country. Unquestionably, Gelei had a major impact on zoology in Hungary which would not be the same without his contributions today.

It is more difficult to do the same thing on a global scale. This is what I will try to do in this brief talk. Although Gelei made major contributions also to the cytology and other aspects of Metazoa, I will restrict this talk to protozoology, befitting the character of this conference. I hope to illustrate Gelei's stature and role with a few selected examples.

József Gelei's scientific career spanned the first half of this century which witnessed two world wars and many economic and social changes. His first major results on the meiotic process were published early in this century and his last papers on limnology and ciliate systematics posthumously in 1954. He made most of his major contributions in the 1920s and 30s, i.e. half a century ago. The conceptual and factual framework of biological sciences, however, has undergone tremendous changes since these times. In Gelei's lifetime the morphological exploration of the protozoan cell, and of cells in general, was limited by the resolving power of the light microscope and their biochemical exploration was in its infancy. Although major contributions to our understanding of the protozoa have been made, the finer morphological and especially the biochemical nature of most structures seen in these cells remained hidden until the advent of electron microscopy and the development of biochemical cytology. A number of specific problems, especially questions concerning the functions of cellular structures have become completely refocused in the intervening decades and some of the most vexing questions of Gelei's time have lost their relevance entirely. It is instructive, however, to see how many of his contributions have still retained their impact.

I will utilize several approaches in this talk. First I will explore the recent impact of Gelei's contributions by citation analysis, then list the taxons named for him, and finally touch briefly upon some of the major topics of his studies.

To evaluate the impact of Gelei's studies in a more or less objective manner, I have resorted to the use of scientometry, although I am not a specialist in it. Table I shows some data culled from Science Citation Index concerning the citation record of Gelei's papers for the past decade and a half, a period representative of contemporary biology. The database of this Index is selective and possibly biased in favor of periodicals with global impact. It is well possible that further citations could be found in more regional publications. On the average about 30 papers by Gelei were cited with 1.5 to 2 citations per article in each of the five year periods examined. The number of articles citing Gelei's papers is of the same order as the number of his papers cited. These data clearly show that his contributions have retained their significance until today in spite of major changes in the outlook of science and mark him as an outstanding scientist of lasting impact. Even if the numbers found are modest, these have to be judged with contemporary citation practices in mind.

Table I

Citations to Articles by J. Gelei

in the Period of 1970-1984*

Number of	1970-4	1975-9	1980-4	1970-84
Articles cited	33	29	19	52
Citations	44	60	33	137 (9.2/year)
Articles citing	27	39	25	91

*Based on Science Citation Index

Table II lists those papers which received several citations during the period examined. These include contributions to most of the topics in which Gelei did original research: meiosis, invertebrate nervous systems, sensory organelles of protozoa, contractile vacuoles, impregnation of protozoa with silver and gold and detection of their "nervous system," effect of nickel ions on ciliates, ciliate systematics and evolution. This list shows that Gelei's work exerts a significant impact in its entirety and not only through one or another single contribution.

It is a tradition in taxonomy that through scientific names one can honor and immortalize one's teacher, mentor, friend, etc. As far as I am aware, Gelei's name was used for three taxons in protozoology. Of his close associates, Stiller (1939) named the peritrich genus *Geleiella* for him. In two instances, however, foreign scientists did honor him this way, showing the wide recognition of his contributions. Kahl (1933), the indefatigable German ciliatologist, created the genus *Geleia* and the family Geleiidae for a group of primitive marine holotrich ciliates. Furgason (1940), an American who did much to clarify the nomenclature of those free living holotrich ciliates which were obtained in bacteria-free cultures by Lwoff (1923), Elliott (1933) and others half a century ago, created the genus *Tetrahymena* for these organisms, and named its type species, *T. geleii*. Furgason justified the use of Gelei's name as follows: "Because we believe that Prof. J. v. Gelei has made some of the most important contributions to our knowledge of these difficult holotrichous ciliates, we wish to give recognition to his work in selecting this species name" (Furgason, 1940, p. 258). Although *T. geleii* subsequently became a junior synonym for *T. pyriformis* (Corliss, 1952), it was in use for about two decades. This period witnessed a logarithmic increase in the number of publications using

Table II

Articles by J. Gelei Cited Four Times or More

in the Period of 1970-1984*

1922. Weitere Studien über die Oogenese des *Dendrocoelum lacteum*. II. Die Längskonjugation der Chromosomen. Arch. Zellforsch. 16, 88-169.

1925. Nephridialapparat bei den Protozoen. Biol. Zentralbl. 45, 676-683.

1928. Nochmals über den Nephridialapparat bei den Protozoen. Arch. Protistenkde. 64, 479-494.

1929. Sensorischer Basalapparat der Tastborsten und der Syncilien bei Hypotrichen. Zool. Anz. 83, 275-280.

1930. "Echte" freie Nervenendigungen. Bemerkungen zu den Receptoren der Turbellarien. Z. Morphol. Ökol. Tiere 18, 786-798.

1931. (with Horváth, P.) Die Bewegungs- und reizleitende Elemente bei *Glaucoma* und *Colpidium* bearbeitet mit der Sublimat-Silber-methode. Magyar Biol. Kutatóint. Munk. 4, 40-58.

1932. Die reizleitenden Elemente der Ciliaten in nass hergestellten Silber- bzw. Goldpräparaten. Arch. Protistenkde. 77, 152-174.

1934. Eine mikrotechnische Studie über die Färbung der subpellikularen Elemente der Ciliaten. Z. wiss. Mikrosk. 51, 103-178.

1935. Ni''-Infusorien im Dienste der Forschung und des Unterrichtes. Biol. Zentralbl. 55, 57-74.

1950. Die Morphogenese der Einzeller mit Rücksicht auf die morpho-genetischen Prinzipien von Severtzoff. Acta Biol. Acad. Sci. Hung. 1, 69-134.

1954. Über die Lebensgemeinschaft einiger temporärer Tümpel auf einer Bergwiese im Börzsönygebirge (Oberungarn). III. Ciliaten. Acta Biol. Acad. Sci. Hung. 5, 259-343.

*Based on the Science Citation Index

T. pyriformis (=*T. geleii*) in studies ranging from molecular biology to protozoan behavior (Corliss, 1965), thus the adjective *geleii* became a household word in biology.

The significance of Gelei's contributions to protozoology received a singular recognition in the monograph The Ciliated Protozoa by Corliss. This volume contains the portraits of six "Twentieth century leaders in the study of ciliates" (Corliss, 1961, Frontispiece and Plate XXII); they are

Alfred Kahl, Emmanuel Fauré-Fremiet, Bruno Klein, József von Gelei, Eduard Chatton and André Lwoff.

In the remaining part of my presentation I will mention briefly a few selected areas of Gelei's protozoological interests. Since his bibliography is reprinted in this volume (Gellért and Müller, 1986), no specific references to his papers will be given. This discussion is not limited to the timespan of the citation analysis. Other speakers at this Conference have provided further details on such topics as silver staining (Paulin, 1986), ciliate systematics (Corliss, 1986) and protozoan evolution (Poljansky, 1986).

Gelei learned his outstanding technical skills in the demanding school of Apáthy, a widely respected master of microscopic techniques, especially of silver staining. He continued the development of various staining processes but the example of his master must have lingered on, because his major technical contributions concern the application of various silver and gold methods to protozoa. With the use of these methods he and his son, Gábor, described a number of different networks of impregnable structures in several ciliates. These have characteristic distributions in the individual species and define in fine detail the various cortical structures of the organisms. Extensive studies have proven the outstanding value of these methods in systematics as well as for the detailed mapping of the ciliate cortex and for following the morphogenetic changes during cell division.

Based on views of Apáthy and others, Gelei assumed that far reaching analogies exist between the impregnated networks in ciliates and fibrils that stain with silver in nerve fibers, thus attributed an impulse conducting role to the ciliate fibrils. With the development of electrophysiological studies the role of the cell membrane in nerve conduction became established and ultrastructural studies identified the fibrils in nerve fibers as skeletal elements. The assumption of conducting function of the ciliate fibrils was disproved in the 1950s, among others by Gelei's student, Béla Párducz (1958b), thus the heated debates of the 1930s on the functional significance of ciliate fibrillar structures are of historical interest only. Ultrastructural studies demonstrated that the networks described by the Geleis correspond to several different structural elements of the ciliate cortex, including ciliary rootlets, adherent walls of cortical alveoles, etc. (Párducz, 1958a).

With the use of his superb technics, Gelei analyzed the structure of the contractile vacuoles of several protozoa, especially of *Paramecium*. In addition to other fine detials, he noted that this structure is surrounded by a specialized region of the cytoplasm, impregnable with osmium, which he named spongiome. Electron microscopic studies revealed that this region consisting of small vesicles is indeed a permanent component of the contractile vacuole (Patterson, 1980). Gelei's term is still in use to designate this structure.

Gelei observed that nickel ions immobilize ciliates partly through suppression of ciliary motion and partly through inducing the shedding of cilia. Nickel treatment is used today in research on various aspects of ciliate physiology, the most important ones being the study of ciliary regeneration (Párducz, 1962) and growth and correlation of feeding processes and cytoplasmic streaming with ciliary activity (Larsen and Nilsson, 1983; Sikora and Wasik, 1978).

Gelei contributed significantly to systematics and described a number of new species, primarily of free-living ciliates. These descriptions are regularly cited. This is not surprising, since contributions to systematics retain their significance permanently and have to be referred to in any revision of the taxon in question. Based on his studies on the morphology of the oral apparatus of *Paramecium*, Gelei recognized that this genus is erroneously included in the order Trichostomatida. First he proposed an intermediate position for this genus and in 1951 he included it in the order Hymenostomatida. His carefully argumented paper remained practically unknown and had no impact because Fauré-Fremiet (1949) arrived earlier independently at the same conclusion.

A major interest of Gelei was the problem of evolution and the comparison of the organizational level and fitness of protozoa and metazoa. He published numerous papers on this topic, mostly in Hungarian, but his definitive paper was also published in German. In this he applied Severtzov's morphogenetic rules to protozoa and reached the conclusion that these rules define the morphogenetic changes not only in metazoan but also in protozoan evolution. Although thought provoking and heuristic, this paper unfortunately has not received the attention it deserves. It is discussed in Dogiel's (1965) General Protozoology but not in most other recent texts. There is a major resurgence of interest in protozoan evolution resulting in a significant expansion of both the available facts and their interpretation. A new evaluation of Severtzov's morphogenetic

principles and of Gelei's ideas on protozoan evolution is definitely a timely task.

This brief summary did not aim at a comprehensive analysis of the significance of Gelei's work. Such an analysis is the task of future historians of science. Our summary, however incomplete, clearly demonstrates that Gelei's contributions represent an active force in contemporary biological sciences. Not surprisingly some of his ideas which he might have regarded as major achievements have become obsolete by now and others which at that time seemed minor survived the test of time. There is no question, however, that Gelei was an outstanding scientist and teacher. It is satisfying to see that half a century after the peak of his activities his name is still alive and well remembered. It is clear that József Gelei earned a place among the great zoologists of our century.

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SCIENTIFIC PUBLICATIONS BY JÓZSEF GELEI*

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1907. Olisthanella hungarica n.sp. Muz. Füz. Kolozsvár, 2, 1-24. (only Hungarian)

1908. Olisthanella hungarica nov. sp.Thesis. Kolozsvár, 1-24. (only Hungarian)

1909. A Szent-Anna tó. /The Szent-Anna lake/ Földr. Közl. Budapest, 37, 177-201. (only Hungarian)

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* This bibliography represents a corrected and slightly modified version of the paper J. Gellért and M. Müller (1954): Die wissenschaftliche Arbeiten von József Gelei. Acta Biol. Acad. Sci. Hung. 5,221-226. Corrections by K.T. Kiss and M. Müller.

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1926. Állati mérgek és gerjesztő anyagok./ Animal venoms and hormones./ Term.-tud. Közl., 58,553-561. (only Hungarian)

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* The author changed the title of this series repeatedly. Contribution VI. and XII. could not be located.

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1934. A csillós véglények (Ciliata) érzőszervecskéi. Die sensorischen Organellen der Ciliaten. Állatt. Közl., 31, 115-138. (in Hungarian with German summary).

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HISTORY

Symposia Biologica Hungarica 33 (1986)

HISTORY OF FREE-LIVING PROTOZOA RESEARCH IN HUNGARY

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In our country the first protozoological paper in Hungarian was published by Tivadar Margó in the middle of the last century. It was written on the Pest-Buda infusorian fauna in 1865 and shows a great knowledge of the literature and gives evidence of extremely keen observations.

The beginning of the systematic protozoological research, however, is linked with the name of Géza Entz Sen. (1842-1919), who was first professor and head of the zoological department of the University of Kolozsvár and later of the Pázmány Péter University in Budapest. His principal work "Studies on Protozoa" which was the result of his systematic investigations, was published in 1888 - at the same time as the basic manuals of Kent, Bütschli, Bronn and Balbiani. He discovered and proved the symbiosis between protozoa and algae. He established a school. Of his pupils not only his son, Géza Entz Jr., but Rezső France obtained world fame. Jenő Daday (1855-1920) was his contemporary. Since Daday's activity may be considered as a chapter of Hungarian hydrobiology, appreciation of his work would be the duty of hydrobiologists. His activity which lasted for nearly forty years was diverse, it included the Carpathian basin and therefore we have got to mention him. He studied the most characteristic groups of water microfauna, among them the protozoa, as thoroughly as anyone after him. We frequently encounter his name in Hungarian and foreign literature.

I have already mentioned that Rezső France was the pupil of Géza Entz Sen. He worked with Entz for six years, at first as a student, later as his assistant. His work on protozoology entitled "Protozoa" was published in 1897, as a separate chapter of a monograph on Lake Balaton. While gathering data to describe the protozoan fauna, he recorded the characteristics of the planktonic and benthic fauna, studied the vertical migration thoroughly, connecting it with environmental factors and considering their correlation. He was an outstanding "ecologist" of his age. His results stimulated E.A. Birge, the well-known American limnologist, as they described not only the phenomena but their causes too. He raises problems and gives impulses to further investigations. (These are mentioned in the obituary written by Birge.)

Géza Entz Jr. was an all-round researcher, but the main field of his activity was protistology. He studied a number of species of marine Tintinnidae in Naples, also a number of fresh-water species in Hungary, made a comparison of them and wrote about his results in a monograph (1906). He stated that the different forms of "lorica" are due to a common type, their development and formation is a sure basis for their classification. While studying the morphology of tintinnids, he drew conclusions on the development of their peristome and their conjugation, which were all new to science. Beside the protozoological investigations, also the up-to-date examinations of Lake Balaton are linked with his name (in association with Olga Sebestyén). Olga Sebestyén is a well-known person of Hungarian hydrobiology and we must not pass over her works. She wrote of the pelagic Ciliata of Lake Balaton. She stated that the density of all oligotrich ciliates increased threefold in the 1950s, and the increase in pelagic Ciliata became such a significant phenomenon which changed the planktonic feature of Lake Balaton.

We may divide Hungarian protozoology in two parts: one period before Gelei and another after him. This second period essentially is the era of his pupils. Gelei was not only an excellent scientist, but an eminent teacher, too.

First I would like to mention Jolán Stiller R., one of his pupils, who became an internationally acknowledged researcher of peritrich ciliates. Would she have written no other work than the Protozoa fauna of the Bátorliget moorland, she would deserve her fame. Nowadays, when our natural resources disappear one after another, thanks to her works something remains from the richness of the territory east of the river Tisza. Her splendid monographs which she wrote on the natron lakes of the Szeged environment and from the Pécsely-bank are the foundations for all young researchers. Her taxonomic volumes, which were edited in the series of Fauna Hungariae, though written in Hungarian are used by foreigners, too, because of their simple, clear drawings. I should like to emphasize that she recognized the transformative effects of the environmental factors before ecology had become fashionable. I may mention, as a proof, her description of the ecotypes of Vorticella microstoma, on the basis of her classical observations. Many species in the literature are named after her in recognition of her work.

The main characteristic of József Gellért's protozoological examinations is their diversity. His works which deal with the ecology and taxonomy of ciliates living under moss or cladonia are among the most frequently cited Hungarian protozoological works. For us who work in the field of Hungarian protozoological research, those ecological statements of his are, I think, the most important ones, together with his examinations of Ciliata which live in the Balaton detritus offshore bar. He had already called attention to the decomposition processes in the detritus offshore bars when Lake Balaton had no water quality problems yet. That was before the littoral region was covered in concrete, an act which removed habitats for organisms which could bring about natural purification. I think, it is necessary to mention that his examinations were complex ones. It is

a pity that, because of the Hungarian language in which he wrote, he - like other excellent researchers - did not receive the deserved international appreciation.

Béla Párducz is the world famous successor of Gelei's physiological-morphological examinations. In his works he examined the basic problems of the ciliary function, the rhythmic movement of cilia, the metachrony of cilia and the changes in the stroke direction. In connection with impulses that coordinate the cilium movement he established a new concept which has neither been verified nor disaffirmed even up to the present. In the field of research on the nervous aspects of tenasmus conduction he surpassed Gelei and became world-famous.

Gábor Gelei walked in his father's footsteps, but his early death prevented him to be successor not only of his father's talent, but also his fame.

János Horváth had a splendid talent for microtechnique and was a keen observer. He soon gave up his main field of research, the hypotrichs. Microbial genetics induced him to leave protozoology, though he began his work with observations of Kahlia simplex individuals without micronuclei.

I must confess that I met the name of József Kormos the first time in Leningrad. Hearing the foreign sound of his name I stated that there was no such Hungarian protozoologist at all. But when I saw his name in written form, I was astonished that they knew the value of our compatriot better than I did. He dealt with Suctoria, a group which is extremely problematic even today. The proper taxonomic place became clarified more than ten years ago. The revelation of productive processes after extremely precise observations is linked with his name. It is to be regretted that he retired very soon and so he was unable to hand down his knowledge to his followers.

Lajos Varga was a prominent contributor to Hungarian protozoology. We can find everything from cave to rainwater spout, from sandy soil to forest floor, from natron lake to Lake Balaton, all examined by him. He was not a pupil of Gelei, but was his contemporary. His name is not so frequently mentioned as that of Gelei, perhaps because he dealt not only with protozoa, but also with other water organisms: for example Rotatoria, Cladocera and Copepoda. He was rather a soil zoologist and hydrobiologist than a protistologist.

The former president of our Protozoological Section, Ferenc Biczók, first made soil protozoological investigations, later examined the photodynamic effects in plasma movement. He was successful in the investigation of the rhizosphere of wheat. Sadly, after initial successes and though the field looked internationally promising, he changed to another theme, in which he made his mark, too. Like other protozoologists, he educated a number of pupils, but none of them is dealing with this branch of science.

We must not forget Zoltán Józsa, who was the researcher of the Tisza, which is the second longest river of Hungary. In the

period of changes in the river's fauna and flora his works, which give a registration of the basic and saprobiological conditions of Ciliata, provide the basis for further investigations.

We always find the past better than the present, but the past of Hungarian protozoology was really a splendid period. It was formed by outstanding persons who gave to the nation and the world knowledge which furthered science.

In our days, mainly those working on free-living protozoa are few, but we are doing our best to keep the reputation set by the fame of Hungarian protozoologists.

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HISTORY OF VETERINARIAN PARASITIC PROTOZOA RESEARCH IN HUNGARY

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In Hungary, the outset of veterinary protozoology may be put at the beginning of the century, when I. Rátz, professor of the Veterinary College began to study the Sarcosporidia, and described a new species from roe-deer. His work was followed by Sándor KOTLÁN, Head of the Laboratory of Parasitology of the Veterinary College, who began to study the coccidial diseases of waterfowl in the nineteen-twenties. This coincided with the establishment of the Department of Parasitology as a separate unit within the Veterinary College. Sándor KOTLÁN was appointed college professor and head of this department in 1935. In the subsequent decades the newly-established department kept publishing a series of new research results in the field of parasitology and parasitic protozoa. Among these, Sándor KOTLÁN' s studies on the intestinal coccidiosis of geese and liver coccidiosis of rabbits were of paramount importance, but the first investigations into the intestinal coccidiosis of chicken in Hungary are also linked with his name.

The development of veterinary protozoology gained a new impetus in the nineteen-fifties, when the staff of the Department of Parasitology of the Veterinary College (now: University of Veterinary Science) was increased, and the Veterinary Medical Research Institute of the Hungarian Academy of Sciences and the Department of Parasitology of the Central Veterinary Institute were called into existence. The staff of the Department of Parasitology of the University of Veterinary Science began to deal primarily with coccidioses of geese and chicken, including the therapy of these diseases. Elaboration and improvement of therapeutic

procedures and testing of drugs and experimental compounds produced either abroad or in Hungary constituted the major part of their work. Among the timely issues of parasitology, research of babesiosis, toxoplasmosis and lamb coccidiosis also formed an important part of the Department's work.

At present, the Department of Parasitology and General Zoology of the University of Veterinary Science is engaged in research of parasitic protozoa of veterinary importance (coccidia, sarcosporidia, Encephalitozoon, etc.), with the aim to determine their incidence in Hungary, epizootiological and pathological significance as well as possible means of control. Recently they have studied the pathological significance of rabbit and poultry coccidioses, elaborated control measures against various species of coccidia, and conducted efficiency tests of anticoccidial compounds. Extensive surveys were conducted to assess the relative incidence in Hungary of Toxoplasma gondii infection, a common concern for both human and veterinary parasitology. The Department of Parasitology has significant achievements in the research of other current topics as well, including the specificity of Eimeria stiedai, therapy of poultry coccidioses, incidence in Hungary of Encephalitozoon infection, and coccidioses of sheep and rabbits. Particularly great efforts were made in the field of chicken coccidioses, with special regard to the large-scale, intensive management conditions. This research continues even today and is focused on the testing and adaptation of new therapeutic compounds, in order to elaborate control measures which would prove most efficient under Hungarian conditions.

The activity of László PELLÉRDY, the most outstanding personality in Hungarian veterinary protozoology, blossomed out in the Veterinary Medical Research Institute of the Hungarian Academy of Sciences. He began his career as a professor's assistant in the laboratory of Sándor KOTLÁN. Already in his early works he paid great attention to problems of veterinary protozoology. His lifework comprises a total of 150 research papers and the world monograph entitled "Coccidia and Coccidiosis" which ran into two editions. The latter has remained a work of fundamental importance in the field of veterinary protozoology. PELLÉRDY's activity and interest covered almost all areas of protozoology. He was as familiar with issues of general biology, zoology and taxonomy as with practical

problems. Among his scientific publications we can find basic works on taxonomy and systematics; he described numerous new species. He studied the coccidioses of nearly all farm animal species. Besides epidemiological and pathological investigations, he conducted detailed studies on the prevention and therapy of coccidioses. His internationally acknowledged expertise is well reflected by the fact that outstanding parasitologists invited him to collaborate in their studies. Always keeping abreast of development, in the final stage of his life and scientific activity he extended his research work to up-to-date ultrastructural, biological and immunological studies which brought about revolutionary changes in our knowledge of coccidia.

In the Veterinary Medical Research Institute of the Hungarian Academy of Sciences, fish protozoology research achieved remarkable progress during the last decade. This research activity, which has by now gained international recognition, started with surveying the parasite fauna of Hungary's fish fauna. This work continues even today, in view of the fact that, as a result of introducing new fish species from far-off continents, new parasite species have appeared in Central Europe. These vigorous research efforts are aimed at elucidating the pathogenicity, epizootiology, developmental cycle and biology of the various parasite species, and at elaborating new preventive and therapeutic approaches. Numerous new disease entities have been described and examined pathologically. The research results obtained on the biology and pathology of myxosporean parasites of fishes are of particular significance.

In addition to its diagnostic and epizootiological work, the Department of Parasitology of the Central Veterinary Institute has published numerous valuable and novel research results. Within the Central Veterinary Institute, workers on the staff of other departments also deal with the study of protozoan infections. The research activity of the working group on protozoan infections of fishes, a group that has been working together for several years, should be highlighted. Significant progress has been achieved in the control of pathogenic protozoa of the honey-bee, and also in the cryptosporodiosis of calves.

The research aimed at surveying the protozoan parasite fauna of Hungary's game stock and at elaborating control measures against them should be mentioned here. Remarkable research has been, and still is, conducted on the intestinal coccidiosis of the brown hare and pheasant, and on the sarcosporidiosis of roe-deer and red deer.

Besides the institutions whose main task is research, a network of regional veterinary institutes and stations has been established. Parasitologists on the staff of these institutions systematically deal with the scientific aspects of practical problems and with the elaboration of control measures.

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PROGRESS IN HUMAN PROTOZOOLOGY IN HUNGARY

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Budapest, Hungary

We have gathered here at the centenary of József Gelei's birth, organized by the Hungarian Academy of Sciences to commemorate a well-known expert in Hungarian protozoology. The delivered lectures seem to have a share in this commemoration. At the same time, since Hungarian protozoology can only be regarded as a whole, a lecture will be presented on the development of human protozoology in Hungary, because some historical events and data appear to be associated with the oeuvre of József Gelei and of some Hungarian scientists working in this field, who were József Gelei's contemporaries.

Human protozoology as a discipline has always been of great importance in Hungary. It is to be noted that only very few of the protozoa that are pathogenic to humans play an epidemiological role. So in the present paper I would like to speak about human diseases, first of all about malaria which has been posing a problem for centuries, as well as about amoebiasis and cutaneous leishmaniasis.

Malaria had been epidemic in Hungary as well as all over the world up to its eradication 25 years ago. Our first written record of it dates from 1778. It was Sámuel Rácz's book published in Buda, in which he described in detail its symptoms and the prescriptions of the quinine preparations used against it (1).

Sámuel Diószeghy, a biologist, in his medical herbal published in Debrecen in 1813 (2) described in detail the preparation and application of Artemisia annua in cases associated with chills, hepatosplenomegaly and jaundice. This meant the

use of the drug named Quing hao su (3) having been applied some years earlier in China, in the hyperendemic areas of Hungary, i.e. in the territory east of the river Tisza and in Transylvania. Artemisia is even now used in Hungary as a flavouring for the aperitif Vermouth.

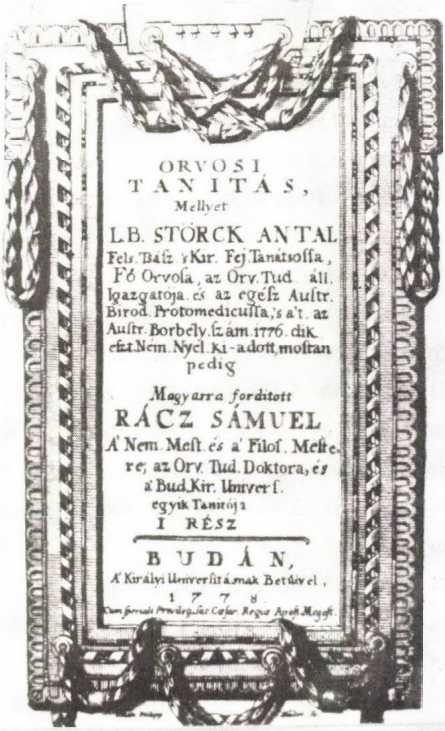
In 1847 Imre Bittner's book was published on 'swamp fever' occurring in the Bánság (Southern Hungary), followed by Hugó Ehrenreich's book on the symptoms and treatment of malaria. Both Bittner, the chief medical officer in Temesvár, as well as Ehrenreich, the clinician, attested by shocking statistical data the severity and insurmountability of malaria (5).

This circumstance prompted Sándor Korányi, the renowned Hungarian physician, to study malaria and TB, these two most prevalent diseases of his time, in his seclusion after the 1848 War of Independence, in which he himself was emotionally involved.

Miklós Jancsó Sen., guided by the same principles, devoted almost 20 years of his life to the research on malaria (6), although under changed conditions, since Sámuel Rácz, Sámuel Diószeghy and Sándor Korányi had not yet known the aetiology of malaria which Jancsó had already been familiar with thanks to Laverán and Ross' discoveries. Miklós Jancsó studied the life-cycle of plasmodia and the mechanism of action of quinine. He was one of the first to deal with the resistance of plasmodium to quinine, attaining his experience at the Kolozsvár University Medical School, in the centre of the hyperendemic territory east of the river Tisza. Miklós Jancsó Jr. did not deal directly with malaria, but rather with protozoology during his RES studies, first of all in his anti-trypanosomal drug research. He was the first to conclude that the resistance of the organism decreases in protozoal infection the importance of which associated with AIDS should be particularly stressed (7).

The fight against malaria gained impetus from the activity of these scientists, first of all from that of József Gelei's contemporaries. Ferenc Lőrincz wrote a book on malaria (8). György Makara and Nándor Zoltai's outstanding activity resulted in the eradication of malaria in Hungary (9, 10). Despite that, similar to other Central European countries, malaria had been

RÁCZ SÁMUEL (1744–1807)


ORVOSI
TANITÁS,
Mellyet
L.B. STÖRCK ANTAL
Fels. Báfz. Kir. Fej. Tanátsossa,
Fő Orvosa, az Orv. Tud. álli.
Igazgatója, és az egész Auſtr.
Birod. Protomedicussa, 's a't. az
Auſtr. Borbely. Iz. ám. 1776. dik.
esz. Nem. Nyel. ki-adott, moſtan
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Magyarra fordított
RÁCZ SÁMUEL
A' Nem. Meſt. és a' Filoſ. Meſte-
re, az Orv. Tud. Doktora, és
a' Bud. Kir. Univerſ.
egyik Tanittója
I RÉSZ
BUDÁN,
A' Királyi Univerſitásnak Betűivel,
1778.
Cum facultate Præſidiali Cæſar. Regiæ Apoſt. Majeſt.

Miklós Jancsó Sen. in 1929

Miklós Jancsó Jr. in 1963

eradicated in Hungary by 1960, the fight against reimplantation of malaria is still going on under strict control and scientific work is to be continued for the following reasons (11).

1. Several thousands of Hungarian citizens working in malaria-endemic areas as well as foreign students studying in Hungary and also tourists may be responsible for importing the disease.

2. The vector is present in our country, and the ecological conditions for the reproduction of plasmodium are also given. Therefore revival of malaria, in principle, is possible at any time. The 137,000 citizens affected by malaria during the 1977 Turkish epidemic, which has not completely been eliminated yet, constitute a special danger. The fact that in the FRG one-and-a-half millions of Turkish workers are travelling across Europe may incur the danger of reimplanting malaria in the entire territory of central Europe including also Hungary. As a consequence, 40 cases of P. vivax malaria were treated at our Institute in the past three years (12).

3. We wish to support WHO and the malaria-endemic developing countries in their fight against the eradication of malaria. How does Hungary contribute to this fight?

(a) All the citizens returning to Hungary or foreign students coming here to study are subjected to obligatory screening examination during which thick and thin blood films are taken which are tested at the Department of Parasitology of the National Institute of Public Health.

(b) Hungarian pharmaceutical factories produce and export a considerable amount of antimalarials. Thirty to 35% of the chloroquine demand of the world is produced by the Alkaloida Chemical Works, Tiszavasvári, Hungary (over 400 tons per year).

(c) Hungarian citizens travelling to malaria-endemic regions have been supplied with malaria chemoprophylactics since the introduction of compulsory screening examinations in 1962. Upon return to Hungary after a long-term service additional screening examinations are performed. The data of 15,600 persons have been assessed. On the basis of our examinations, it was found that chemoprophylactics (chloroquine, pyrimethamine) supplied by our Institute and taken regularly by our patients

provide sufficient protection against the manifest disease. Assessing the data of 12,780 persons, it was stated that with regular chemoprophylaxis the incidence of malaria was one-tenth of that in persons not taking the drug (1, 2 or 12%) (13).

An account of our experience with chemoprophylaxis has been given in a separate lecture (14).

(d) Investigation of chloroquine-resistant cases by using the in vitro drug-sensitivity test of Rieckmann and Antauanou. The chloroquine-resistant P. falciparum in Tanzania was described by us in 1980 (15).

(e) Tropical medicine including malaria has been introduced into the undergraduate and postgraduate training of Hungarian and foreign students and physicians.

(f) The first Hungarian university handbook on tropical diseases and parasitology was published in 1973. The third edition is in press (16).

Our further tasks are being performed in cooperation with WHO.

1. Studies on postmalarial exposure in persons returning from malarious areas (17).

The Hungarian Tropical Health Institute will assess the postmalarial exposure of persons having stayed in malarious areas for varying lengths of time, in addition to the microscopic diagnosis of malaria, also by serological methods, and will carry out the following investigations.

(a) All persons are asked about their previous medical histories to obtain data, such as the patients' age, sex, place of residence, duration of stay in the malarious area, the country where he had been staying, and previous malarial attacks and their treatment.

(b) Blood samples are taken from all persons returning from malarious areas for immunofluorescent antibody test.

(c) A total of 700 retrospective examinations have been performed so far. IgG was found in 23 cases, IgM in 5 cases among persons having taken regular prophylaxis but with no manifest disease (13).

2. The second contract between the Hungarian Tropical Health Institute and WHO for new potential antimalarial com-

Table 1. Imported cutaneous leishmania cases

Endemic area	Number of cases	Time of infection					
		1981		1982		1983	
		month	cases	month	cases	month	cases
Algir	3	08	2	07	1	-	-
Iraq	20	-	-	07-09	14	02-09	6
Kuwait	1	-	-	-	-	02	1
Libya	4	09	1	08	1	09-10	2
Syria	3	09	2	-	-	08	1
Total	31		5		16		10

Fig. 1

pounds (18) contains the following tasks: (i) the de novo development of antimalarial drugs, (ii) the development of already available drugs.

These tasks are performed in cooperation with the Department of Organic Chemistry, Kossuth Lajos University, Debrecen and the Department of Pharmacology, University Medical School, Debrecen.

The de novo synthesis and screening of potential antimalarial compounds includes (i) the primary synthesis of potential antimalarial compounds, (ii) primary efficacy screening of selected compounds, (iii) primary toxicity screening, (iv) further efficacy testing using P. falciparum in vitro and P. berghei screening of various resistance patterns in the mouse.

Further toxicological testing will be carried out with all compounds which were found to be effective in the P. falciparum in vitro screening and/or against drug-resistant P. berghei. This test includes (i) repeating of acute toxicity in mice, (ii) acute toxicity in rats and (iii) subacute toxicity in mice and rats.

The tasks are being performed with the hope of finding a new antimalarial compound.

Besides, within the framework of CMEA, we are taking part in the activity of committees for the fight against parasitological diseases, first of all malaria.

The Hungarian Tropical Health Institute seems to be the main coordinator of research on antimalarial drugs.

Amoebiasis and toxoplasmosis constitute a growing epidemiological problem in Hungary. In view of the fact that lectures will be presented on amoebiasis and toxoplasmosis, I would like to speak only about cutaneous leishmaniasis. In Hungary, neither autochthonous nor imported cutaneous leishmaniasis cases had occurred up to 1981. Between 1981 and 1983, 31 Hungarian subjects with cutaneous leishmaniasis were treated in the clinical department of the Hungarian Tropical Health Institute (19) (Table 1 and Fig. 1). This is due to the fact that currently thousands of Hungarian citizens are working in Mediterranean and Near-Eastern endemic countries. Our patients, whose skin lesions occurred in different parts of their bodies, underwent special examinations.

The smear taken from the base of the ulcerous skin lesions was stained with Giemsa solution for 10 minutes after being fixed, and the pathogen was examined at a magnification of 400 or 1000 under a light microscope. Only those cases were considered positive where Leishmania tropica had been found either

Table 2. Therapy of cutaneous leishmaniasis

Drug	Number of cases	Recovered	Improved	Unchanged
Delagil (chloroquine)	6	3	-	3
Tubocin (rifampicine)	6	2	4	-
Lomidin	3	1	1	1
Klion (metronidazole)	2	-	-	2
Stibophen	3	1	2	-
Glucanhine	4	2	2	-
Hibernal* (chlorpromazine)	7	7	-	-

*Chlorpromazine was applied locally simultaneously

in intracellular or extracellular form in severe new infections.

Beside the usual anti-protozoal drugs, chlorpromazine was used which, on the basis of literary data, can be regarded as a fairly new therapeutic method. Chlorpromazine (Largactil or Hibernal) was used partly locally and partly systemically. The skin lesions of our patients treated with chlorpromazine healed within 3 to 6 weeks, in a shorter time than by using other compounds. Neither side-effects nor a second attack of the disease were found during additional examinations.

In my lecture I intended to summarize the developments and present activity in human protozoology in Hungary.

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HISTORICAL REVIEW OF STUDIES ON FREE-LIVING PROTOZOA IN JAPAN

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It was in 1877 when "the Biology Course" was launched for the first time in Japan; this was in the College of Science of the Imperial University of Tokyo (now called Faculty of Science, Tokyo University). Two years later, the Biological Department was divided into 2, i.e. Zoological and Botanical Institutes. At this point it is worth mentioning that those who wanted a chance to be a protozoologist, without exception, had to study in the Zoological Institute for 44 years until 1921, when the 2nd zoological course started in the College of Science at the Imperial University of Kyoto, now Kyoto University. The first professor of zoology was not a native man. Perhaps, a similar case can be found everywhere in developing countries. In the case of Japan he was an American naturalist named E.S. Morse (1877-1879), then animal anatomist C.O. Whitman succeeded in this position from 1879 to 1881. In 1881, 3 students became the first alumni, but no one studied Protoza as their graduation thesis. Of these 3 graduates, Iijima became an associate professor soon after graduation and there was no professor in the Zoological Institute since Whitman had returned to America. In 1882, K. Mitsukuri was appointed the first Japanese professor. Because he had finished his postgraduate course in 1881 at the Johns Hopkins University under the guidance of Dr. Brooks, who was one of the friends of Mr. Morse, Prof. Mitsukuri remained in this position until 1909. In this year, just after the time when Ishikawa completed the zoology course, he went to Prof. Weismann at Freiburg and Iijima also went to Germany under Prof. Leuckart. At the "Medical" College of the Imperial Uni-

versity of Tokyo, now Faculty of Medicine, Tokyo University, Dr. F.F. Hilgendorf had taught biology from 1873 to 1876. He made some collections of Mollusca, Annelida, Crustacea and Pisces mainly in Hokkaido and Enoshima. In 1876 Dr. H. Ahlburg became a professor as successor to Hilgendorf from 1876 to 1878, when Dr. Döderlein came to Japan as the third professor of zoology. During the period 1878-1881, he collected many animals, and published the results in 1882 under the title of "Faunistische Studien in Japan" (Arch. Naturges. 49). Unfortunately this paper did not deal with "Protozoa". The first paper on Protozoa appeared in 1874, the animal was Fusulina japonica, a fossil Foraminifera. Unfortunately again, it was not presented by a Japanese but a German named W. Gümbel and it was not in the Japanese Journal but the German "Ausland". In 1885, Tôkio Seibutsu Gakkwai (Biological Society of Tokyo) separated into Tôkio Shokubutsu Gakkwai (Botanical Society of Tokyo) and Tôkio Dôbutsu Gakkwai (Zoological Society of Tokyo). According to the late Prof. H. Ohshima (1959) the period from 1877 to 1885 could be regarded as the first generation, because 'general biology' was introduced exclusively by American or Americanized teachers. He regarded the period from 1886 to 1910 as a second generation, since it is characterized by the fact that most zoologists were direct pupils of the professor who belonged to the first generation, or 'grandpupils' of Mr. Morse or Mr. Whitman. In fact, Iijima, the first pupil of Mr. Morse, was professor of zoology from 1886 until 1921. The potential zoologists were therefore strongly affected by him. The 1st Marine Biological Station of Japan was established in 1887 at Misaki, Kanagawa-ken on the repeated recommendation from Prof. Morse, Prof. Mitsukuri, et al. Consequently, members of the Zoological Institute of Tokyo were more interested in marine (cf. Noctiluca) than freshwater animals. The first zoological publication appeared in 1888 under the name of "Dôbutsugaku Zasshi" (Zoological Magazine) by the Tôkio Dôbutsu Gakkwai (Zoological Society of Tokyo) organized in 1878, and contains 2 protozoan papers: Mitsukuri, K.: An easy way of obtaining Amoeba (in Jap.), No. 1: pp. 27-28; Ono, N.: An easy way of obtaining Radiolaria (in Jap.) No. 2: p. 54. At this time, the content of the paper was not original

but rather instructive. Perhaps, I am permitted to say ca. 30% of the content was occupied by the review or introductory papers from foreign sources or re-examination. In fact, Prof. Mitsukuri himself serialized "Lectures on Zoology" (in Jap.) in this magazine, Vol. 1, in the following way: Amoebida: pp. 87-92, Heliozoa & Radiolaria: pp. 117-120, Flagellata, Suctoria & Ciliata: pp. 147-152, Ciliata & Sporozoa: pp. 187-192.

In 1890, the Fisheries Department was opened at the College of Agriculture of the Imperial University of Tokyo (now Faculty of Agriculture, Tokyo University) and Ishikawa became a professor. But no pupils became protozoologists. From 1888 to 1910, other papers on protozoa are as follows: Shishido, I. (1889): Phosphorescence of Noctiluca. Zool. Mag. 1: 305-306; Colour of Noctiluca. Ibid. 1: 394; Oka, A. (1889): Light from Noctiluca. Ibid. 1: 394; Ishikawa, C. (1890): Notes on Noctiluca. Ibid. 2: 325-327, 374-378; Goto, S. (1892): How the test is formed in the freshwater Rhizopoda. Ibid. 4: 69-70; Some experiments by cutting protozoa. Ibid. 4: 213-218, 351-356, 390-393, 453-469; Saito, J. (1893): Ciliata. Ibid. 5: 61; Takakura, U. (1895); Digestion process in Infusoria. Ibid. 7: 227-228; Ishikawa, C. (1896): Ephelota from Misaki. Ibid. 8: 195-196; Note on the Jap. species of Volvox (in Eng.). Ibid. 8: 25-37; Yasuda, A. (1897): On the accommodation of some infusoria to the solutions of certain substances in various concentration (in Eng.). Annot. Zool. Japon. 1: 23-29; Takakura, U. (1897): Central mass in Heliozoa. Zool. Mag. 9: 113-114; Miyajima, A. (1898): Vital staining of Protozoa. Ibid. 10: 92-95; Ishikawa, C. (1898): On Noctiluca. Ibid. 10: 273-283, 352-358, 365-369; Nishikawa, J. (1900): Red tid. Ibid. 12: 127-133; Yatsu, N. (1900): Trichodina. Ibid. 12: 190-191; (1903): Two observations on Amoeba. Ibid. 15; Fujita, S.: Planktonic ciliates from Shinobazu-ike pond. Ibid. 17: 185-186; Yatsu, N. (1907): Effect of volcanic eruption on plankton. Ibid. 19: 128-129; Fujita, S. (1908): On the genus Didinium. Ibid. 20: 71-77; Kawamura, T. (1908): Effect of alcohol on velocity of binary fission in Infusoria. Ibid. 20: 167-168; Koidzumi, M. (1909): Amoeba. Ibid. 21: 199-217, 243-255.

In my consideration, the third period from 1911 to 1924 is a transitional one towards the fourth period. This is because (1) in 1925 the 1st Annual Meeting of the Zoological Society of Japan was held at the Tokyo Imperial University; (2) two famous protozoologists, Mr. Abe, T., a teacher of Ishii and Kanno of Hosei University and Mr. Kamada, a teacher of Kinoshita of Tokyo University, graduated in this year; (3) the first zoological graduates appeared in 1924 both in Kyoto Imperial University (Profs. Ikeda, Kawamura, & Komai) and in Kyushu Imperial University (Prof. Oshima) preparing for a new period; (4) almost all professors in this time were taxonomists and embryologists of Invertebrata and therefore much interested in systematic zoology. In the third period, the following papers appeared: Kasuga, K. (1912): Fusion experiment in Amoeba limax. Zool. Mag. 24: 314; Ishibashi, E. (1912): Heliozoa decorated with spicules. Ibid. 24: 418; Ishikawa, H. (1913): Wundheilungs- u. Regenerationsvorgänge bei Infusorien. Arch. Entw. 35; Edmondson, C.T. & Kingman, R.H. (1913): Notes on Japanese Protozoa. Zool. Mag. 26: 49; Kanda, S. (1914): On the geotropism of Paramecium. Ibid. 26: 221; Nagasawa, R. (1916): X-ray treatment of Foraminifera. Ibid. 28: 239; Izuka, A. (1916): Response to light in Paramecium. Ibid. 28: 510; Iijima, I. (1918): A Manual of Zoology (in Jap.), pp. 211-250 for Protozoa. Dainihon Tosho Co. Ltd., Tokyo; Kawamura, T. (1918): Freshwater Biology (in Jap.). A few pages for free-living protozoa, cf. Hydrobiological Station was stablished in Otsu, Shiga-ken in 1914; Endo, Y. (1918): Green-colored Noctiluca. Zool. Mag. 30: 30-31; Kanda, S. (1918): Further studies on the geotropism of Paramecium. Ibid. 30: 266; Mori, O. (1919): On Arcella. A graduation paper at Tokyo Imperial University. From 1920 to 1924, we do not have any remarkable papers on free-living Protozoa, but I think it will be better to mention that at least 4 events occurred in this period: (1) Yatsu who was only interested in experimental morphology and physiology became a professor of zoology from 1922 until 1938; (2) the biological course was opened at the College of Science of Tohoku Imperial University, Sendai, in 1923; (3) Tôkio Dôbutsu Gakkwai (Zoological Society of Tokyo) developed into the Nihon Dôbutsu Gakkwai (Zoological Society of

Japan) in 1923; (4) the most renowned systematic zoologist, Uchida, T., graduated from Tokyo University. In my consideration again, the years from 1925 to 1954 could be regarded as the fifth period and the years from 1955 to this year to be the sixth. But, so far as these periods are concerned, it is clear that most papers were written in English or German, occasionally in French, and circulation of the papers was much better than in the previous periods. So, I should like to close this short report just mentioning 13 facts:

1) In 1927, Goto, Sasaki and Esaki participated at the 10th International Congress on Zoology held in Budapest.

2) In 1929, the Bunrika Daigaku of Tokyo (afterwards Tokyo Kyoiku Daigaku and now Tsukuba Daigaku) and that of Hiroshima (now Hiroshima Daigaku) were established.

3) In 1930, the Zoological Institute was opened at the College of Science, Hokkaido Imperial University, Sapporo (Prof. Uchida, T.).

4) In 1943, Keitô Dôbutsu Gaku (Systematic Zoology) Vol. 1 was published. Yoken-Do, Tokyo, pp. 91-294 for Protozoa by Miyashita and K. Morishita.

5) In 1946, the Biological or Zoological Departments were opened at every national university.

6) In 1948, Genseidôbutsu (Protozoa) was published by Fukui, T. Tosui Shobo, Tokyo, 154 pp.

7) In 1949, the Biology Institute was opened at Osaka University.

8) In 1953, the 1st Symposium on Protozoa was held in Kyoto, attached to the 24th Annual Meeting of the Zoological Society of Japan.

9) In 1962, the Society of Protozoology was established in Tokyo by Dr. Abe (Hosei University) and Dr. Yagiu (Hiroshima University). Dôbutsu Keitô Bunruigaku (Animal Systematics). Ed. Uchida. Vol. 1 appeared. Nakayama Shoten, Tokyo, pp. 83-327 for Protozoa by Yagiu.

10) In 1968, Gensei Dôbutsu Gakkai Shi (Japanese Journal of Protozoology). Vol. 1, No. 1 was published.

11) In 1972, revised edition by Uchida of Dôbutsu Bunruimei Jiten (Dictionary of Animal Taxonomy by Yatsu) appeared. Nakayama Shoten, Tokyo, pp. 27-94 for Protozoa by Yagiu.

12) In 1981, Gensei Dôbutsu Zukan (Illustrated Encyclopedia of Protozoa). Edited by Inoki, with the co-operation of 44 specialists was published. Kodansha Scientific, Tokyo, 816 pp.

13) In 1984, the Zoological Magazine and Annot. Zool. Japon. were united into one journal named the Zoological Sciences and chemical or molecular treatment became dominant in most fields of zoology.

ADVANCES IN FREE-LIVING PROTOZOA RESEARCH

I. TAXONOMY, METHODOLOGY, PHYSIOLOGY

Symposia Biologica Hungarica 33 (1986)

JÓZSEF GELEI'S WORKS ON REGULARITIES OF PROTOZOAN EVOLUTION AND THE CURRENT STATE OF THE PROBLEM

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The outstanding Hungarian biologist József Gelei has made an important contribution to the solution of both zoological and general biological problems. Particularly well known are his studies on Turbellaria and Protozoa (primarily ciliates). Among other things, J.Gelei and his disciples (notably B.Parducz) performed a series of investigations of the intimate structure of the ciliary and fibrillar apparatus in ciliates. These works, carried out at the light-optical level, formed the basis for subsequent electron-microscopic research and demonstrated that ciliates display a most complex organization. This enabled Gelei to compare the protozoans, and, in particular, the ciliates with whole multicellular organisms rather than with single metazoan cells. Gelei wrote (1950, p.125): "Auf Grund unserer Forschungsergebnisse sind wir vollkommen berechtigt, den Einzeller im Spiegel des Vielzellers zu betrachten"...(On the basis of our research data we are perfectly justified to consider the unicellular /being/ as a mirror image of a multicellular one...).

It is natural that such an approach to the protozoans as whole (and rather complex) organisms remaining at the unicellular level led Gelei to problems concerning the general regularities of their evolution, as compared with those of metazoans. Obviously, he was concerned with the level of evolutionary transformations which is now called macroevolution.

Morpho-physiological regularities of evolution were extensively studied in the thirties through the fifties by Sewertzoff, Rensch, Huxley, Remane and other specialists in the

theory of evolution. Among these works, of primary importance were the studies of Sewertzoff summarized in his monograph "Morphologische Gesetzmässigkeiten der Evolution" (1931). It was this book that directed Gelei's attention to the problem of morpho-physiological regularities in evolution at the cellular level of organization, that is, in protozoans. The result of his research and meditations was his paper entitled "Die Morphogenese der Einzeller mit Rücksicht auf die morphogenetischen Prinzipien von Sewertzoff" (1950). In this essay, which is of great interest for both protozoology and theory of evolution, Gelei starts from his original concept about the nature of Protozoa, and, using a vast protozoological material (chiefly on ciliates), considers the problem of morpho-physiological regularities of their evolution. The main and very significant conclusion drawn by Gelei is that the essential principles (modi) of morpho-physiological evolutionary transformations, established by Sewertzoff for the metazoans, are equally applicable to the protozoans, and especially to their highest group - the ciliates. Intensification of functions, their weakening, phase fixation, change of functions, extension of functions, and other pathways of transformation of the organs (in all 13 modi), postulated by Sewertzoff for the metazoans, were convincingly demonstrated by Gelei to occur in protozoans as well. Thus, they really operate also at the unicellular level of organization. Gelei did not confine himself to a description of the ways of evolutionary changes in protozoans which corresponded to those already established by Sewertzoff, but postulated several new modi of evolutionary transformations applicable to both Protozoa and Metazoa. In Gelei's opinion, most important among them is the phenomenon of combination of functions and/or organs, e.g., fusion of their ducts and cavities. Rather significant is also the process of neoformation of organs (or organelles), for instance, the development of a complex digestive apparatus in various groups of ciliates.

The above work of J.Gelei is of great significance since it involves the Protozoa in the general system of morpho-physiological regularities of evolution. Later, Gelei's ideas

were developed further by the Polish protozoologist Z.Raabe (1971), who, using a wealth of new factual material obtained mostly on ciliates, extended the basic principles of Gelei.

Along with the studies by Gelei and Raabe who primarily stressed the similarities between the morpho-physiological regularities of the evolution of eukaryotic unicellular organisms and those of multicellular animals, there arose another trend that treats the problem of protozoan evolution from a different point of view. Without any rejection of the above concepts, this line of thought stresses primarily the differences in the regularities of the evolution between the Protozoa and the Metazoa. This trend was started by V.Dogiel with his important paper "Polymerisation als ein Prinzip der progressiven Entwicklung bei Protozoen", published in 1929. When studying the morphological regularities of evolution, Dogiel took into account some quantitative indices, namely, the number of homologous organs or organelles. Extensive material pertaining to almost all phyla of the Metazoa and concerning various systems of organs was used by him to show that in most metazoans progressive evolution involves oligomerization (decrease in the number) of homologous organs, correlated with their morphological (as well as histological) progressive differentiation (see: Dogiel,1954). Numerous poorly differentiated organs commonly arise at the initial stage of evolution (e.g., numerous small ganglia on the ventral nerve cord of annelids), and then they decrease in number. This is accompanied by complication of the structure of the remaining organs (ventral ganglia in various Arthropoda). These regularities, established by Dogiel, are now widely used in comparative anatomical and evolutionary studies and are being elaborated by many investigators.

Unicellular eukaryotic organisms (protozoans) are subject to quite different regularities of evolutionary changes of the number of organelles and parts of the cell as compared with those concerning the metazoan organs. In protozoans, the progressive morpho-physiological evolution involves polymerization (increase in number) of organelles and parts of the cell. This postulate, first advanced by Dogiel (1929), was later extended

and developed in a series of papers, primarily by Soviet authors (Poljansky,1971,1981; Poljansky and Raikov,1972). During progressive evolution of protozoans, polymerization may show a great diversity and involve various parts of the protozoan body.

Polymerization of the cytoplasmic organelles, which does not involve the nuclear apparatus, has occurred in many protozoan taxa. Among the flagellates, the number of kinetids and related organelles may increase. This is particularly conspicuous in flagellates inhabiting the intestine of termites and wood-eating cockroaches (Trichonympha, Teratonympha, etc.). If one assumes that ciliates have originated from some flagellates, which is universally acknowledged, the development of their ciliary apparatus on the basis of a relatively small number of flagellate's kinetids would give another excellent example of progressive evolution linked to polymerization.

In evolution of such a vast and, on the whole, progressive group as the Foraminifera, the leading role was played by polymerization of some cytoplasmic components - the locomotory apparatus, the skeletal elements, owing to the appearance and development of multilocularity. Of no less importance was polymerization of the cytoplasmic components in evolution of a large group of marine free-living protozoans, the radiolarians. Here it involved the skeletal elements, the particular fibrillar apparatus of the Acantharia - their axopodial myofrisks, etc.

Polymerization of mitochondria, Golgi bodies, sometimes of contractile vacuoles, etc. can be found in all large protozoan taxa.

Ciliates of the family Ophryoscolecidae, inhabiting the rumen of ungulate mammals, provide a striking example of progressive evolution based on polymerization. During a relatively short period of time (in the geological sense of the word, since their hosts, the ruminants, make a comparatively "young" group), these ciliates covered a long path of progressive evolution from the simple-structured genus Entodinium to the complex genera Polyplastron, Ophryoscolex and some others. This case is of special interest since here, as it seems, the suc-

cessive stages of phylogenetic transformations remain extant. This might be due to the peculiarities of the habitat, for the rumen is extremely rich in nutrients.

Along with cytoplasmic structures, the polymerization frequently involves the nuclear apparatus. This is commonly expressed in multinuclearity. The examples are numerous and widely known: the multinucleate amoebae Pelomyxa and Chaos, the multinucleate flagellates Calonympha, the vast group of Opalinida, and many others.

Multiplication (polymerization) of the number of genomes in protozoan evolution can be brought about also without polymerization of the nuclei themselves: it can occur via an increase (due to DNA replication) of the number of genomes within a single nucleus. This results in polyploid nuclei, the formation of which is thus another kind of polymerization. Such progressive evolutionary changes are well exemplified by the radiolarians Phaeodaria, which can have more than 1000 genomes per nucleus at certain stages of the life cycle (Grell, 1953). The chromosomes belonging to each genome here remain interconnected end to end in a chain-like manner, thus maintaining the integrity of the individual genomes.

Most complex is the evolution of the nuclear apparatus in ciliates, which is associated with differentiation of two qualitatively distinct nuclei - the micronucleus (generative nucleus) and the macronucleus (the vegetative one). This typical nuclear dualism apparently arose within the phylum Ciliophora and could have been preceded by polymerization of the nuclei, some of which became differentiated into vegetative nuclei (macronuclei). This is likely because some lower ciliates (order Karyorelictida) still have many nuclei, some of which are micronuclei, while others are macronuclei but with a DNA content similar to the diploid one. The subsequent evolution of the macronucleus took the path of polyploidization. The overall amount of DNA in the macronucleus can reach a very high level. The macronuclear DNA content in some ciliate species may exceed over 5000-fold that of the micronucleus (assumed to be diploid). These facts made it possible to interpret the macronucleus as a highly polyploid nucleus of exceptional

transcriptive activity. Recent studies, however, show that the question is not that simple. The development of the macronucleus proved to be accompanied not only by polyploidization of the nucleus through replication of genomes, but also by profound qualitative rearrangements of the genomes. A part of the chromatin is eliminated from the developing macronucleus, and at least some genes, including the ribosomal RNA genes, are intensively replicated (amplified). In some ciliates (e.g., in the Hypotrichida) the chromosomal organization of the genomes is completely lost (Raikov, 1978,1982; Raikov and Ammermann, 1976; Poljansky,1974).

The leading role of polymerization, operating at various structural levels from molecular to organellar in progressive macroevolution of the protozoans, does not rule out the existence of an inversely directed process, that of oligomerization (decrease in the number of cell components). This is most distinctly pronounced in higher protozoans, the ciliates. Examples of this phenomenon are numerous. After polymerization had accompanied the formation of the ciliary apparatus, its subsequent evolution often involved various kinds of oligomerization. This may be partial reduction of the ciliary apparatus associated with specialization of movement. An example of this is Didinium, in which the somatic ciliature is restricted to only two ciliary crowns due to reduction of a considerable portion of the ciliature. The decreased number of kineties on the ventral side of many crawling forms (e.g., Hypotrichida) is another example. Oligomerization by fusion of individual cytoplasmic components into higher order structures is widespread among the ciliates. For instance, fusion of cilia into such structures as cirri, membranes, membranelles etc. is common.

Let us draw some conclusions. The studies of J.Gelei and Z.Raabe have shown that the basic principles and pathways of transformation of organs in evolution, established by Sewertzoff for the metazoans, are applicable also to the unicellular eukaryotic level of organic evolution. This is a most significant conclusion for both protozoology and general theory of evolution.

Along with these similarities, there are essential qualitatively unique features of macroevolution in unicellular eukaryotes as compared with multicellular organisms. These features, revealed mainly by protozoologists of the Soviet school, consist first of all in different roles taken by polymerization and oligomerization in evolution. They have been illustrated above with actual examples. A question arises as to what is the cause of these differences. Why does polymerization of homologous structures play the leading (though not exclusive) role in progressive evolution of protozoans, unlike the metazoans? We (Poljansky and Raikov, 1977) advanced a hypothesis that these differences are determined by the level of structural organization of the respective components: on one hand, these are organelles or parts of the cell, on the other hand, these are complex multicellular structures (organs). The rigidity and stability of the macromolecular structure of organelles strongly reduces the possibilities of complication of their structure; hence, the only way leading to intensification of the functions which is available to protozoans is an increase of the number of organelles. The situation is different with the organs of Metazoa. Here, wide possibilities are available for complication and differentiation of their internal structure, which creates conditions for the appearance, in the course of morpho-physiological progress, of a few highly differentiated organs.

József Gelei, whose centenary we are now celebrating, was the first to start investigating the morpho-physiological regularities of evolution in unicellular eukaryotic organisms. In this way, he made a considerable contribution to the development of both protozoology and theory of evolution.

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THE DEVELOPMENT OF CILIATE SYSTEMATICS FROM THE ERA OF JÓZSEF GELEI UNTIL THE PRESENT TIME*

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While the 8,000 species of ciliates represent only 6-7% of all protists described to date, their phylum -- the Ciliophora -- is the fourth or fifth largest among the 45 comprising the kingdom Protista, with only the foraminifers and the diatoms (largely through their fossil forms) greatly outnumbering them (Corliss, 1984). And it is widely recognized that the ciliates represent one of the most compact and circumscribed groups, with all subgroups clearly united phylogenetically and perhaps with no living close relatives among other known protist assemblages. Nevertheless, the world of ciliate systematics has not been a static one; and it is my intention here to review briefly not so much the actual taxonomic expansion and the shifts in or within schemes of classification of these unique organisms as the bases for such changes and revisions. In so doing, we shall discover that the oft-overlooked contributions of such precise workers as the late Professor József Gelei, remarkable in his combined talent of ecologist and cytologist, served as a major factor in bringing about a "new look" at "old" problems.

FIRST PERIOD IN CILIATE SYSTEMATICS

In brief review (see Corliss, 1974a, for details), we may recall that the first period in the history of ciliate systematics can be said to have formally commenced with the monumental work on the Protozoa by Bütschli, that great architect of protozoology, published in several volumes during the 1880's. With little refinement, his scheme for the ciliates dominated the literature for more than half-a-century; and, indeed, its influence is

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still powerful in some quarters today. The basis for separating the 8-10 subgroups then recognized (see Table 1) was, reasonably enough, essentially the easily visible -- with the microscopes of the time -- differences in the location and/or composition of the external ciliature, somatic and/or oral. It was not difficult to conclude that, in general, the simpler-appearing species comprised orders "lower" on the phylogenetic/evolutionary scale than the orders that contained forms with the most elaborate, most complex, ciliature. Thus, the so-called gymnostomes, or naked-mouthed forms, were considered as very likely the most primitive ciliates and hypotrichs or peritrichs, the most evolved. Recall that only some 500 species were known a hundred years ago.

Table 1. Bütschlian Classification

Class I N F U S O R I A
Ciliata
HOLOTRICHA
Gymnostomata
Trichostomata
Astomata
SPIROTRICHA
Heterotricha
Oligotricha
Hypotricha
Peritricha
Suctoria

Giants in ciliatology in those early and exciting days during the Age of Discovery, themselves preceded by such unforgettable pioneers as O. F. Müller, Ehrenberg, Dujardin, Claparède & Lachmann, Wrzesniowski, and perhaps above all, Friedrich Stein, included -- besides Otto Bütschli -- Schewiakoff, Kent, Wallengren, Maupas, G. Entz, Sr., Stokes, and Delage & Hérouard. Names of still other appropriate cytologists and microscopists could be cited (see Corliss, 1978-79), many of them attached to or trained in the great German universities, with Bütschli's own laboratory at Heidelberg a foremost center surpassed in due time only by that of Richard Hertwig, at Munich, himself a student of the celebrated evolutionary biologist and early protistologist Ernst Haeckel of Jena.

SECOND PERIOD IN CILIATE SYSTEMATICS

I have suggested (Corliss, 1974a) that the second period in ciliate systematics, the Age of Exploitation, might be considered to have extended from 1930 to 1950. If I may be allowed the liberty, here, of altering that span slightly -- to 1925-1954 (which is more accurate, anyway) -- it will, by neat coincidence, cover exactly the period of time during which József Gelei produced his own set of most influential publications on the cytology, taxonomy, and ecology of various species of ciliates. Thus, we need to reexamine this period in a little more detail.

Interestingly enough, the titular leader in the taxonomy of ciliates at

this time, the prolific (15 monographs in nine years) Alfred Kahl of Hamburg, did not really alter the overall classification system of his predecessors. It remained basically (only) a fleshed-out Bütschlian scheme: see his culminating series, Kahl (1930-35), and Table 2. But the all-important expansions were the direct outcome of the significant papers of primarily a small band of leaders, including a modest Hungarian of note in ciliatology, a man who studied in R. Hertwig's laboratory and whose memory we are honoring in the special International Conference convened here in Budapest this week (see Frontispiece). With Gelei, as students and/or nearby colleagues, we must also mention the names of Biczók, P. and J. Horváth, Párducz, Stiller, and Szabó -- and Gelei's son Gábor -- already active producers before the middle of the present century (with later appearance of such additional researchers as Géllert and J. and K. Kormos; and, today, Bereczky and others carry on the fine tradition).

Table 2. Kahlian Classification

Subphylum C I L I O P H O R A
- **Protociliata**
  - OPALINATA
- **Euciliata**
  - HOLOTRICHA
    - Gymnostomata
      - Prostomata
      - Pleurostomata
      - Hypostomata
    - Trichostomata
    - Apostomea
    - Hymenostomata
    - Thigmotricha
      - Stomodea
      - Rhynchodea
    - Astomata
  - SPIROTRICHA
    - Heterotricha
    - Ctenostomata
    - Oligotricha
    - Tintinnoinea
    - Entodiniomorpha
    - Hypotricha
  - PERITRICHA
    - Mobilia
    - Sessilia
  - CHONOTRICHA
- **Suctoria**

Workers from other countries deserving inclusion in this elite group were Chatton & Lwoff, Collin, and Fauré-Fremiet of France; Penard of Switzerland; Klein of Austria; Beers, Calkins, Hall, Hegner, Jahn, Kidder, Kofoid, Kudo, Metcalf, Noland, and Wenrich of America; Bresslau, Doflein & Reichenow, Grell, Hartmann, Matthes, Stammer, and Wetzel of Germany; Jírovec and Šrámek-Hušek of Czechoslovakia; Dogiel, Gajewskaya, Poljansky, Strelkow, and Swarczewsky of the U.S.S.R.; Wang and Nie of China; Yagiu of Japan; H. and Z. Raabe of Poland; and Lepşi of Roumania. Some of these investigators and many of their students also played important roles in subsequent periods in the history of systematic ciliatology.

Importance of Ecological Observations

One of the two major emphases in the ciliophoran studies of this second

period was the attention given to the ecology of the forms under investigation. Recognizing the importance of the natural habitats of ciliate populations also led to the discovery of many new species. The 500 of Bütschlian times quickly grew to more than 3,000, in large measure due to the careful uncovering of previously neglected niches. This is beautifully exemplified in the great series of 16 studies published by Gelei -- over a period of 18 years -- under the general title of "Beiträge zur Ciliatenfauna der Umgebung von Szeged" (e.g., see Gelei, 1932a, the first such study, and Gelei, 1950b, the last). And a separate tripartite ecological monograph, appearing posthumously, contained a major treatment of 75 species of ciliates found in the specialized fresh-water habitat of temporary pools: see Gelei (1954). Such researches were thorough, including observations on feeding behavior, encystment, general physiology*, and cytology [for techniques, see below], as well as taxonomy. A number of the species described were new to science.

It is encouraging to note, today, a return to Gelei's approach to protozoan ecology, working with living material in its natural habitat. References to pertinent works of such a kind are most conveniently found in the bibliography of ecological research (period 1910-1981) on fresh-water and terrestrial protozoa published recently by the Freshwater Biological Association of Great Britain (Finlay & Ochsenbein-Gattlen, 1982); and, as an outstanding very recent example, see Foissner (1984). The whole book, now in press, by Dragesco & Dragesco-Kerneïs (1985) deserves special ecological citation for its thorough coverage of all habitats, including marine and estuarine. One of the roles of taxonomy, to serve as handmaiden to ecology, is not to be neglected (Corliss, 1980).

Importance of Cytological Techniques

The second Geleian emphasis of lasting value was his insistence on precision in microscopical observations, including care in the staining of fixed material (Gelei, 1928a). It is most interesting, historically, that the several techniques of silver impregnation -- a method that was destined to revolutionize the whole fields of systematics and phylogenetics of ciliates -- all arose, essentially independently, within a very few years of

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* His excellent series on "sense-organs" of ciliates was ahead of its time and demands re-attention today (see Gelei, 1933, 1934d, 1938b, 1939). He also studied the contractile vacuole system in some detail (e.g., see Gelei, 1925, 1928b, 1934a, 1938a,c), his microanatomical observations never yet surpassed.

one another!* In 1926, Klein (1926) introduced his quick-and-easy "dry" technique, which became very popular despite some drawbacks to its usage; in the same year, Rio-Hortega (1926) developed a method for silver-staining fibrillar epithelial tissue that eventually (e.g., see Fernández-Galiano, 1966) became adapted to ciliate material; and four or five years later there appeared the improved "wet" methods of Chatton & Lwoff (1930) and of Gelei & Horváth (1931; and see Gelei, 1932b).

Interestingly enough, widespread taxonomic application and conceptual appreciation of the cytoarchitectural revelations of these silver methods did not occur until after Gelei's death, although Kahl quickly showed interest in the new characters becoming available and Gelei (1952) himself, just before his passing, used the new information in a phylogenetic/systematic treatment of the then large (sub)order Trichostomata. Incidentally, Gelei (1950a) also was impressed by the morphogenetic principles of Sewertzoff; this might have led him to applying such concepts to problems in ciliate systematics had he lived a few years longer.

THIRD PERIOD IN CILIATE SYSTEMATICS

The stage was now set for a "new look" at the ciliated protozoa. Ecological and cytological data of comparative value in taxonomy were rapidly being accumulated, and the morphological diversity of forms being discovered was lending itself to phylogenetic and evolutionary considerations. So, a third period in ciliate systematics followed hard upon the second, in fact overlapped with it; and -- as the Age of the Infraciliature -- it "rose to its heights on the wings of silver" (Corliss, 1974a), the technique to which pioneer Gelei and co-workers, as well as Klein and Chatton & Lwoff (and subsequently Fauré-Fremiet and his many followers), had contributed so much. Light microscopy still dominated the cytological scene, although works on ciliates by cell biologists utilizing transmission electron microscopy shortened the period dramatically. While earlier (Corliss, 1974a) I set the time-span for this Age of the Infraciliature as 1950-1970, it might be better, now, to consider its ending in about 1963, the year of the publication of Pitelka's (1963) landmark book on electron microscopy of protozoa, a fitting formal introduction to the fourth period or the Age of Ultrastructure, treated on later pages.

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* It is true that the now very popular, and excellent, Protargol technique, applicable to both flagellates and ciliates, did not come into general usage until the 1950's and even later: see Kirby's (1950) widely cited description of it. But it stemmed from the earlier technical paper by Bodian (1937).

During the third period, the number of species had doubled (again), that is, risen from 3,000 to 6,000; new habitats were being explored (e.g., the interstitial zone for free-living forms and the intestinal tracts of scores of new hosts for symbiotic species); and new techniques, or refinements of old ones, were being developed. The classification scheme appeared to be similar; at least, it occupied only about the same number of lines (see Tables 2 and 3). But what had changed dramatically was the conceptual basis for the phylogenetic arrangement of the taxonomic groups comprising the Ciliophora. Although data of the past were largely used, their interpretation was often strikingly different. The heuristic ideas of such French leaders as Chatton and Fauré-Fremiet (and their students) were startlingly new in comparison with the Bütschlian/Kahlian concepts.

Table 3. Faurean Classification

Subphylum C I L I O P H O R A

Class CILIATA

Holotricha
- GYMNOSTOMATIDA
  - Rhabodophorina
  - Cyrtophorina
- SUCTORIDA
- CHONOTRICHIDA
- TRICHOSTOMATIDA
- HYMENOSTOMATIDA
  - Tetrahymenina
  - Peniculina
  - Pleuronematina
- ASTOMATIDA
- APOSTOMATIDA
- THIGMOTRICHIDA
  - Arhynchodina
  - Rhynchodina
- PERITRICHIDA
  - Sessilina
  - Mobilina

Spirotricha
- HETEROTRICHIDA
  - Heterotrichina
  - Licnophorina
- OLIGOTRICHIDA
- TINTINNIDA
- ENTODINIOMORPHIDA
- ODONTOSTOMATIDA
- HYPOTRICHIDA
  - Stichotrichina
  - Sporadotrichina

We need to consider, albeit briefly, the bases for this refreshingly "new look" at ciliate classification, a view modestly set forth by Fauré-Fremiet (1950) in a succinct but now classical paper published 35 years ago. A convenient expansion of it, in the form usually cited in the literature, is that found in reviews of mine (Corliss, 1956, 1961) and in the once-popular Honigberg Report (Honigberg et al., 1964). Essentially, the underlying hypotheses were these: that the infraciliature of all ciliates (particularly the kinetosomes) has an integrity and continuity of its own; that the mode of fission separates flagellates from ciliates; that there is homology in the "mouthparts" of ciliates belonging to widely separated groups; that morphogenetic phenomena, including stomatogenesis, and polymorphism offer characteristics of both taxonomic and evolutionary value; and that similar-appearing adaptive organelles may represent only the outcome of convergent evolution. Gelei

and other perceptive earlier workers had foreseen some of these principles, but it remained for the French cytologists and protistologists (plus such outstanding newcomers to the field as Lom of Czechoslovakia and Wolska of Poland) to fully appreciate their significance and to apply them to the construction of phylogenetic trees and to improved "natural" systems of classification. It should be noted that neo-Kahlian schemes were also published during this time by Russian (Cheissin & Poljansky, 1963; Poljansky & Cheissin, 1962) and Polish (Raabe, 1964a,b) workers, but they were relatively conservative in their modifications.

Subsequent revisions of the new schemes of classification of this productive third period, however, were -- by hindsight -- destined to come about quickly, primarily because rapid accumulation of still newer data, particularly via electron microscopy, was continually obliging researchers to reevaluate taxonomic conclusions drawn practically the day before. Fauré-Fremiet himself was outstanding in reacting wholesomely to the impact of exciting new and often more precise information -- in fact, he was often the discoverer of such fresh data! The sadness or reluctance felt by many of us, however, stemmed primarily from the attractive "teachableness" of the compact scheme then in vogue (see again Table 3) and its apparent "uncomplicatedness," a convenience short-lived, alas. As the numbers of species climbed, once again, by several hundreds and as the separateness (= assumed evolutionary diversity) of the different subgroups became clearer, a further splitting of formerly thought-to-be cohesive units became inevitable. And so arrived the Age of Ultrastructure, with its attendant complications for the poor taxonomist -- not only in ciliatology but in the whole of protistology (Corliss, 1985).

FOURTH PERIOD IN CILIATE SYSTEMATICS

Although I have proposed, above, the year 1963 as the beginning of the fourth period in taxonomic ciliatology, the first major revisions in the scheme of classification did not appear until 1974, three years after the passing of Fauré-Fremiet. There is always a lag in the adoption of anything new, of course; by the time, for example, that protozoological textbook writers replace my (Corliss, 1961) Faurean system with my recent fourth-period one (see Corliss, 1979a), the latter itself will probably have been superseded by fresher ones based on such works as those by Small & Lynn (1981, 1985): see below. No longer can a single period be represented by a single phylogenetic tree or classification scheme. Today, we're still in

the Age of Ultrastructure; but it's likely to be overlapped in due time by an Age of Molecular Biology, as more sophisticated technologies become of service in protist systematics.

We need to pause here and ask, once again, what is the rationale in promulgating still newer phylogenetic/systematic arrangements of the subgroups of ciliates, members of that protist phylum so noted for its relative homogeneity? Are newer data so different that they are having an impact requiring major taxonomic revisions? And/or are fresh interpretations, newer concepts, largely responsible for the seemingly relentless drive to revise? Are the ciliates themselves all that different from what they appeared to be in the times of Gelei, Kahl, and Fauré-Fremiet? Must students and nonspecialists (from molecular bench biologists to field ecologists) be driven nearly to despair when they confront the seemingly ever-changing world of ciliate systematics?!?

It is clearly evident that ultrastructural data are continuing to oblige "new looks" at the homologous or nonhomologous nature of many minute structures or organelles beyond the limits of light microscopy (Corliss, 1979b). The vast majority of today's 8,000 species of ciliates have yet to be subjected to the ultramicrotome; there's no doubt in my mind but that exciting new discoveries will be made that will serve as a legitimate basis for rethinking some of our past taxonomic decisions. One quite recent example may be mentioned, the revelation that the century-old "homokaryotic ciliate" Stephanopogon -- so vital to an aspect of Raikov's (1969, 1982) beautiful hypothesis on the evolution of the dual nuclear apparatus unique to the Ciliophora and to the "eociliate" theory in Corliss' (1974a,b, 1979a) systematic scheme for the phylum -- must now be recognized as a flagellate that, like the opalinids, is far removed from the ciliate phylogenetic tree (Lipscomb & Corliss, 1982). Stephanopogon, perhaps most closely related to the euglenid and the bodonid groups of flagellates (Corliss, 1984), represents neither the first nor the last case of a radical shift in the taxonomic position of a protist following careful restudy of its cytoarchitecture. In fact, one of the most significant revelations in this context was made by Gelei (1934b), without the benefit of electron microscopy, when he recognized that the "well known" ciliate Paramecium was not a trichostome species but, in effect, a hymenostome, a discovery not really appreciated until many years later (Corliss, 1961). While ultrastructural data are underlying the unity of ciliates, particularly by their confirmation of the integrity of the infraciliature (long ago foreseen, for example, by Chatton & Lwoff,

1935; Fauré-Fremiet, 1950; Gelei, 1932b, 1934c), it is true that they are also justifiably emphasizing significant differences at ultra-levels, differentiations that need to be reflected in proposed phylogenetic interrelationships of the forms or groups of forms being compared. Inevitably, this leads to an inflation of taxa, even at suprafamilial levels. With the imminent advent of a flood of data from molecular biological investigations (see Corliss, 1985, and references therein), we can anticipate recognition of still more evolutionary divergence among various lines of ciliates, once again probably requiring expansion in the taxonomic hierarchy as well as revision of past schemes (for example, see Meyer et al., 1985).

Another approach that, today, is serving as a basis for revision in classification systems among all assemblages of protists (e.g., see Heath, 1981; Lipscomb, 1985), not just ciliates, involves methodologies of analysis of data (see Smith & Patterson, 1985, for an even-handed, up-to-date review). The Computer Age is (also) upon us! Being able to handle large quantities of comparative data -- the Constellation of Characters Hypothesis of the author (Corliss, 1976) -- via computer programming represents a boon to the whole field of taxonomy. The most recent application of such methodology to ciliate systematics is the study by de Puytorac et al. (1984), but also see the earlier similar but less ambitious work by Lynn (1979).

But, certainly, the principal reason for the recent rash of revisions in the "macrosystem" of the ciliates is related to differing interpretations of data and/or different emphases, conceptually, concerning the significance of various organelles and other ultrastructures characteristically found throughout diverse taxa within the phylum. This is the last topic to be reviewed here, and I shall attempt to present an unbiased account of the ideas and rationales behind the several major classification schemes proposed within the past five or six years. [Space restrictions demand a brevity that may make my treatment seem over-simplistic: for this I apologize.]

CURRENT SYSTEMS OF CLASSIFICATION

The four systems chosen for brief comparison are those published -- in chronological order -- by Corliss (1979a), Jankowski (1980), de Puytorac et al. (1984), and Small & Lynn (1985). An overview of apparent differences -- although perhaps misleading in some respects, without the explanations too lengthy to present here -- may be gained by contrasting the numbers, names, ranks, and relative positions of the highest-level taxa listed in Tables

4-7. Incertae sedis groups have not been included. Whereas Corliss and the French school have approximately the same number of suprafamilial categories, Small & Lynn have nearly double the number and Jankowski triple.

Incidentally, the very interesting macrosystem of Seravin & Gerassimova (1978), stemming from the seminal publication of Gerassimova & Seravin (1976), is not discussed in the present paper, primarily for two reasons. Aside from the important and startling revelation of the widespread occurrence of the postciliodesmal fibers (stacks of postciliary microtubular ribbons) among various subgroups of ciliates, the Russian authors develop neither an extensive rationale nor a detailed taxonomic system for the Ciliophora overall. Secondly, the value of their discovery has been both recognized and partially incorporated into the schemes of the other classifications considered below. Furthermore, the use of only one or two characters -- presence or absence of the postciliodesma, presence or absence of the kinetodesma -- is inadvisable in view both of the non-universality of these features (or else their overlapping) in various ciliate taxa and of the forced neglect of other salient characters surely also of major significance in the evolutionary history of the phylum.

The Macrosystem of Corliss (1979a) [see Table 4]

Precursors of this are found, in effect, in Corliss (1974a,b) and even in de Puytorac et al. (1974) in which any differences are relatively minor. Its adoption -- or support -- has been widespread, notable major publications including Curds (1982), Curds et al. (1983), Dragesco & Dragesco-Kerneïs (1985), Levine et al. (1980), Madrazo-Garibay & López-Ochoterena (1985), and Raikov (1982). The rationale involved, in what is mostly an expanded refinement of the later ideas of Fauré-Fremiet, stresses the desirability of using multiple characters from many fields (karyology, oral and somatic morphology, morphogenesis [fission and modes of stomatogenesis], ecology, behavior, life cycles, adaptations to sessility, light- as well as electron-microscopy, biochemistry). However, some conclusions drawn on the basis of "intuition," wittingly or otherwise, may be too subjective to withstand the scrutiny of time. And certain new data demand a number of changes. The greatest handicap in ascertaining with assurance the monophyletic nature of the major taxa -- true for the following systems as well, of course -- is our most distressing dearth of data on the majority of the species under consideration! As our knowledge expands, revision will thus be inevitable.

Table 4. Corlissian Classification (version of 1979*)

Phylum C I L I O P H O R A

Class KINETOFRAGMINOPHORA

Gymnostomata
- KARYORELICTIDA
- PROSTOMATIDA
  - (3 suborders)
- HAPTORIDA
- PLEUROSTOMATIDA

Vestibulifera
- TRICHOSTOMATIDA
  - (2 suborders)
- ENTODINIOMORPHIDA
- COLPODIDA

Hypostomata
- SYNHYMENIIDA
- NASSULIDA
  - (2 suborders)
- CYRTOPHORIDA
  - (3 suborders)
- CHONOTRICHIDA
  - (2 suborders)
- RHYNCHODIDA
- APOSTOMATIDA
  - (3 suborders)

Suctoria
- SUCTORIDA
  - (3 suborders)

Class OLIGOHYMENOPHORA

Hymenostomata
- HYMENOSTOMATIDA
  - (3 suborders)
- SCUTICOCILIATIDA
  - (3 suborders)
- ASTOMATIDA

Peritricha
- PERITRICHIDA
  - (2 suborders)

Class POLYHYMENOPHORA

Spirotricha
- HETEROTRICHIDA
  - (6 suborders)
- ODONTOSTOMATIDA
- OLIGOTRICHIDA
  - (2 suborders)
- HYPOTRICHIDA
  - (2 suborders)

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*Order Primociliatida, created for Stephanopogon (see earlier page of text), has been dropped here.

The Macrosystem of Jankowski (1980) [see Table 5]

To a degree, precursors of this are to be found in many preceding papers and monographs by this prolific and innovative ciliatologist: for example, see Jankowski (1967, 1972, 1973, 1975). On the other hand, his schemes of classification have changed considerably over the years, at least superficially and from a nomenclatural point of view (see appropriate tables in Corliss, 1979a, and contrast them with the present Table 5). Stressing ecological factors, structures visible by light microscopy, life cycles, and especially morphological and functional characters associated with the oral area, Jankowski's latest macrosystem is still at considerable variance with other modern arrangements and requires deeper analysis than can be offered here. A prodigious and logical worker, his ideas must not be ignored -- but they need to be better understood. His inflationary tendencies have not gone unnoticed: many of his orders or suborders (totaling 157 if incertae sedis taxa are counted!) contain but a single genus.

Table 5. Jankowskian Classification (version of 1980*)

Phylum C I L I O P H O R A

Class APICOSTOMATA

Telostomata
PROSTOMATIDA
(5 suborders)
CYCLOTRICHIDA
DIDINIIDA
BURSELLOPSIDA
HOMALOZOONIDA
ORTHOVESTIBULIDA
DIDESMIDA

Rhynchostomata
DILEPTIDA

Prionostomata
STEPHANOPOGONIDA

Class PLEUROSTOMATA

Antostomata
TRACHELOCERCIDA

Pleurostomata
AMPHILEPTIDA
LOXODIDA
(2 suborders)

Symbiophagina
KENTROPHORIDA

Class RIMOSTOMATA

Stichofragmina
PLATYOPHRYIDA
BALANTIDIIDA
(2 suborders)
COLPODIDA

Contofragmina
PLAGIOPYLIDA

Class SYNCILIOSTOMATA

PARAISOTRICHIDA
BLEPHAROCORYTHIDA
(2 suborders)
ENTODINIIDA
(2 suborders)

Class SPIROTRICHA

HETEROTRICHIDA
(6 suborders)
BURSARIIDA
ARMOPHORIDA
ODONTOSTOMATIDA
PERITROMIDA
LICNOPHORIDA
PLAGIOTOMIDA
HYPOTRICHIDA
(7 suborders)
OLIGOTRICHIDA
(2 suborders)
TINTINNIDA

Class CYRTOSTOMATA

Clinostomata
SYNHYMENIIDA
PRONASSULIDA
NASSULIDA
MICROTHORACIDA
(2 suborders)

Hypostomata
HYPOSTOMATIDA
(3 suborders)

Class CHONOTRICHIDA

EXOGEMMIDA
CRYPTOGEMMIDA

Class SUCTORIA

Tomogenea
PODOPHRYIDA
METACINETIDA
PARACINETIDA
URNULIDA
ALLANTOSOMATIDA
OPHRYOCEPHALIDA
TACHYBLASTONIDA
EPHELOTIDA

Vermigenea
SPELAEOPHRYIDA
DENDROSOMIDIDA
THECACINETIDA
OPHRYODENDRIDA
(3 suborders)

Endogenea
ACINETIDA
(2 suborders)
TRICHOPHRYIDA
DENDROSOMATIDA
(2 suborders)
PSEUDOGEMMIDA
ENDOSPHAERIIDA

Evaginogenea
DISCOPHRYIDA
DENDROCOMETIDA

Neotenea
CYATHODINIIDA

Class HYMENOSTOMATA

Stichostomata
TETRAHYMENIDA
(2 suborders)
APOHYMENIDA

Scuticostomata
LOXOCEPHALIDA
(3 suborders)
PLEURONEMATIDA
(5 suborders)
HYSTEROCINETIDA
(2 suborders)
PARASTOMATIDA

Astomata
ASTOMATIDA
(4 suborders)

Class APOSTOMATA

FOETTINGERIIDA
COLLINIIDA
CHROMIDINIDA
PHTHOROPHYRYIDA
CONIDOPHRYIDA
ASKOELLIDA

Class PERITRICHA

FIBRODISCIDA
(12 suborders)
DENTODISCIDA
(2 suborders)

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* Incertae sedis orders (eight) and suborders (seven) have been omitted.

The Macrosystem of de Puytorac et al. (1984) [see Table 6]

Although this surprising fresh scheme from the French school has its data-base roots in many earlier studies by de Puytorac and colleagues (including the major expositions by de Puytorac & Grain, 1976, and de Puytorac et al., 1976), it differs substantially from the one published a decade ago by the Clermont-Ferrand group (de Puytorac et al., 1974), at least at the level of the highest taxa within the phylum. Some five dozen specific

Table 6. Classification of French School (version of 1984*)

Phylum C I L I O P H O R A

Subphylum **Karyorelictophora**

Class KARYORELICTEA

KARYORELICTIDA
(2 suborders)
PLEUROSTOMATIDA
(2 suborders)

Subphylum **Kinetophragminophora**

Class GYMNOSTOMEA

Gymnostomia
ARCHISTOMATIDA
(2 suborders)
TRIMYEMIDA
PROSTOMATIDA
(3 suborders)
SPATHIDIIDA

Astomia

Class PHYLLOPHARYNGEA

CYRTOPHORIDA
(2 suborders)
SUCTORIDA
RHYNCHODIDA

Subphylum **Hymenophora**

Class COLPODEA

COLPODIDA
(4 suborders)
CYRTOLOPHOSIDA

Class HYPOSTOMEA

Hypostomia
PENICULIDA
NASSULIDA
(2 suborders)
PARAHYMENOSTOMATIDA
(or PARAPENICULIDA)

Class PERITRICHIA

PERITRICHIDA
(2 suborders)

Class HYMENOSTOMEA

Hymenostomia
HYMENOSTOMATIDA
(3 suborders)
APOSTOMATIDA

Scuticociliatia
SCUTICOCILIATIDA
(3 suborders)

Class SPIROTRICHEA

Hypotrichia
HYPOTRICHIDA
(3 suborders)
CLEVELANDELLIDA

Heterotrichia
HETEROTRICHIDA
(3+ suborders)

Oligotrichia
OLIGOTRICHIDA
(2 suborders)

* Authors included great majority but apparently not all groups in this scheme.

(and "representative") genera were studied, determining phenetic relationships (Legendre, 1979) by computer analysis of 122 characters. Seventy of the selected features, almost all ultrastructural and kinetidal in nature, related to the "somatic cortex" of the ciliates examined; 39, to the oral area ("buccal cortex"); six, to features of stomatogenesis; five, to the nuclear apparatus; and two, to the type of fission (binary or budding). The choice of characters, the size of the data-base, and some of the nomenclatural decisions seem somewhat inadequate or unjustified, but the overall approach warrants serious consideration and emphasizes the potential value of numerical treatment of multiple features to produce presumably (if the characters are simple logical units) unbiased phenograms or cladograms (when a cladistic approach is employed).

The Macrosystem of Small & Lynn (1985) [see Table 7]

This well thought-out and logical system had its direct foundation in Small & Lynn (1981), with conceptual input from such preceding studies as Lynn (1976, 1979, 1981), Lynn & Small (1981), and Small (1976). While, once again, using many of the same data as employed in the preceding classifications, interpretation of what characters or character-systems have greatest information content of phylogenetic importance has led to quite different -- sometimes very startling! -- groupings of the more than 200 families comprising the phylum Ciliophora. Claiming that previous authors, especially Corliss (1979a), have placed undue emphasis on characteristics of the oral ciliature and of the infraciliature at the light-microscopical level, Small & Lynn stress what they term "primary structural features," particularly the allegedly highly conserved somatic cortical ultrastructures. They conclude that it is at the level of the "organellar complex" that patterns meaningful in evolutionary considerations emerge. This Structural Conservatism Hypothesis (Lynn, 1976) was derived from careful study of a great many observations by transmission electron microscopy, and it represents the cornerstone of their phylogenetic assumptions and the resulting scheme of higher classification. In agreement with Corliss (1974a,b) and others, these authors also endorse Raikov's (1969) thought-provoking concept concerning the evolution of nuclear dualism, thus accepting the karyorelictid ciliates as "corticorelicts" representing ancestral ciliate stock. Aspects of morphogenesis are considered important, too. But many of the characters treated as of highest-level taxonomic significance by Corliss, Jankowski, and others are of value, according to Small &

Table 7. Classification of Small & Lynn (version of 1985)

Phylum C I L I O P H O R A

Subphylum **Postciliodesmatophora**

Class KARYORELICTEA

PROTOSTOMATIDA
LOXODIDA
PROTOHETEROTRICHIDA
PROTOCRUZIIDA

Class SPIROTRICHEA

Heterotrichia
HETEROTRICHIDA
(2 suborders)
CLEVELANDELLIDA
PLAGIOTOMIDA
ARMOPHORIDA
PHACODINIIDA
LICNOPHORIDA
ODONTOSTOMATIDA

Choreotrichia
CHOREOTRICHIDA
(3 suborders)
OLIGOTRICHIDA

Stichotrichia
STICHOTRICHIDA
(3 suborders)

Subphylum **Rhabdophora**

Class PROSTOMATEA

PROSTOMATIDA
PRORODONTIDA

Class LITOSTOMATEA

Haptoria
HAPTORIDA
PLEUROSTOMATIDA
PHARYNGOPHORIDA

Trichostomatia
VESTIBULIFERIDA
ENTODINIOMORPHIDA
(3 suborders)

Subphylum **Cyrtophora**

Class PHYLLOPHARYNGEA

Phyllopharyngia
CYRTOPHORIDA
(3 suborders)
RHYNCHODIDA
(2 suborders)

Chonotrichia
EXOGEMMIDA
CRYPTOGEMMIDA

Suctoria
EXOGENIDA
ENDOGENIDA
EVAGINOGENIDA

Class NASSOPHOREA

Nassophoria
SYNHYMENIIDA
NASSULIDA
(2 suborders)
MICROTHORACIDA
PROPENICULIDA
PENICULIDA
(2 suborders)

Hypotrichia
EUPLOTIDA
(2 suborders)

Class OLIGOHYMENOPHOREA

Hymenostomatia
HYMENOSTOMATIDA
(2 suborders)
SCUTICOCILIATIDA
(3 suborders)

Peritrichia
SESSILIDA
MOBILIDA

Astomatia
ASTOMATIDA

Apostomatia
APOSTOMATIDA
ASTOMATOPHORIDA
PILISUCTORIDA

Plagiopylia
PLAGIOPYLIDA

Class COLPODEA

CYRTOLOPHOSIDIDA
BRYOPHRYIDA
COLPODIDA
BURSARIOMORPHIDA

Lynn, only at lower levels within the overall scheme. While "curing" some polyphyletic faults of predecessors, have certain of their own groups become paraphyletic? They have made a most commendable attempt to retain preexisting nomenclature whenever possible for their major taxa.

General Conclusions

Despite such differences in the numbers and arrangements of ciliate taxa as those notable in the various tables of this paper, I believe that all modern systematic ciliatologists will agree with the following general conclusions: (1) Growth of our knowledge has brought about better appreciation of the great diversity within the phylum, and thus an inflationary expansion of the early schemes of classification is justifiable (whether pedagogically attractive or not). (2) Many species from many taxa have been inadequately studied to date, and careful comparative researches on them (with emphasis on ultrastructural approaches) may lead to substantial changes in current systems. (3) Biochemical, genetic, and especially molecular information is badly needed and, once obtained on a sufficiently large scale, may well dictate alteration of some of our views on phylogenetic interrelationships within the phylum, thus requiring still further revision of our present tentative schemes. (4) Logical, numerical methodologies -- defensible and verifiable -- should be employed in order to handle the growing masses of pertinent data in an unbiased, totally scientific manner. (5) Nevertheless, the models of precise cytological studies and extensive ecological observations set for us by such incomparable leaders in the first half of this century as József Gelei still serve as indispensable guides for all future work in ciliate systematics.

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* By no means are all of Gelei's papers cited in this brief ciliatological bibliography. His scientific productivity resulted in some 150 publications (see Géllert & Müller, 1954) -- with thus an average of nearly five works per year -- and his contributions on ciliates alone represent slightly over half of that impressive total.

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SOME PROBLEMS OF AMBIREGNAL TAXONOMY AND A POSSIBLE SOLUTION

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Ambiregnal taxonomy is a new term and relates to that area of taxonomy which concerns itself with the classification of taxa that fall under the jurisdiction of more than one code of nomenclature.

Most taxa are located within a hierarchical scheme of classification that is entirely regulated by one of the nomenclature codes. This type of relationship between classification and nomenclature is here referred to as "coherent". Exceptions typically involve organisms the affinities of which to plants, animals or bacteria are arguable.

There are three common ways in which more than one code of nomenclature may come to be applied to a taxon. Firstly, some taxa may have been treated always, for the purposes of nomenclature, as plants by some and as animals by others (e.g. euglenid flagellates, collar-flagellates and dinoflagellates.) As the botanical and zoological codes are independent, homonyms may be formed (Urceolus is the name of a euglenid and a fungus; Triadinum is the name of a ciliate and of a dinoflagellate). In some cases, one homonym may be unacceptable. If the codes are followed to the letter, they may require the formation of synonyms. An example is Peranema, a name for a genus of euglenid flagellates which is the valid name under the rules of the zoological code (ICZN). The name is illegitimate under the botanical code (ICBN) as it is preoccupied by a name for a fern. A second name, Pseudoperanema, is required under the ICBN for the euglenid genus (Silva, 1980a).

A second reason why two sets of rules may be applied to one taxon is the arbitrary, but entirely legal, annexing by workers employing one code, of organisms traditionally named in accordance with a different code. Examples are the inclusion of unicellular xanthophytes within the

classification of protozoa (Levine et al., 1980) or the treatment of trypanosomes and other kinetoplastid flagellates as algae (Silva, 1980a).

The final reason why two codes might be applied to one taxon is that an improved understanding reveals that its closest affinities are with organisms which traditionally have been subject to a different code of nomenclature. A natural classification would aim to place together nomenclaturally incompatible bedfellows.A familiar example is the proposal to transfer the blue-green algae from the jurisdiction of the botanical code to the bacteriological code (Stanier et al., 1978).

It is the third category which is of most concern to protistologists. Recent ultrastructural studies have led to proposals that certain types of protozoa are related to, and should be classified with, various types of algae. Phylogenetic links have been argued between actinophryid heliozoa and pedinellid algae (Patterson & Fenchel, 1985), opalinids and heterokont algae (Patterson, 1985) or euglenids and kinetoplastids (Cavalier-Smith, 1981). Such proposals may lead to integrated schemes of classification which pay scant regard to traditional nomenclatural boundaries (Cavalier-Smith, 1981; Corliss, 1984).

A variety of solutions has been proposed to deal with the emerging difficulties, most of which are problems of homonymy (Corliss, 1983; Ride, 1982). These, and further suggestions were collated by J.O. Corliss and presented at the 3rd International Congress of Systematic and Evolutionary Biology, at Brighton (July, 1985). The proposals do not all deal with the same issues nor are they mutually exclusive. Mostly, they retain the traditional practice of coherence between nomenclature and classification. This paper presents an option of non-coherent classification for discussion.

Options under discussion include the development of a single unified code or of a new code for protists. There is insufficient movement in these directions by the regulating bodies of the ICZN, ICBN and the ICNB for these solutions to meet present needs. The second proposal further suffers from being tied to a paraphyletic taxon and the taxa to which the code should apply would be a source of debate. The option would be superfluous if non-coherent taxonomy was adopted.

Jeffrey (1981) has argued for the arbitrary allocation of higher taxa to existing codes. This is indicated above as being a source of difficulties. It either demands a break with traditional nomenclatural

practice in order to cluster closely related taxa, or if traditional practice is retained so are numerous polyphyletic or paraphyletic taxa.

The botanical and zoological codes of nomenclature have rules which permit taxa named under a different code to be incorporated within them (Rules 2a and 10f of the ICZN, and 45.4 and 65 of the ICBN). This offers a means of identifying problems with each name and resolving them individually. An example of this approach may be found in Silva (1980b). Solutions typically require the name to satisfy both codes. This solution is unrealistic in requiring an effective knowledge of both codes of nomenclature and of a wide variety of organisms. The movement of taxa with a very large number of species (such as the foraminifera) to an area regulated by another code (in order to maintain a natural classification) would generate an enormous amount of work and make this option impracticable. It probably would be resisted by most specialists on that group.

In addition, Corliss has identified an urgent need for a guiding body which would seek to resolve the problems of ambiregnal taxonomy, including those associated with the flood of new names for high-level taxa.

The option I wish to propose here is that coherency be relinquished and that individual authors apply the code of their choosing to the taxa under consideration. It is presumed that they would elect to use that code under which the majority of subfamilial names were proposed. However, unlike the option for the arbitrary assignment of taxa to codes, coherence between classification and nomenclature is not required nor expected. The adoption of a code for one taxon would not oblige other workers to apply the same code to sister taxa, subtaxa or taxa of higher rank. Resulting classification would include a mixture of botanical and zoological taxa.

Two fragments of what such classifications might look like are presented below. These are offered for explicit discussion and not for adoption. This approach has many advantages. No new codes are needed. There is no break with tradition of nomenclature of taxa at lower rank. This approach is very flexible. As perceptions of genealogical relationships change, taxa can be moved to a new location, but without any associated change in nomenclature. The integrated and non-partisan nature of this proposal would reduce the desire to annex taxa.

Fragment 1	Fragment 2
Division Heterokontae	Class Actinomonadea
.	Order Pedinellales
.	Family Pedinellaceae
Class Chrysophyceae	Genus *Pedinella*
Class Phaeophyceae	Genus *Pseudopedinella*
Class Bacillariophyceae	Genus *Apedinella*
.	Family Actinomonadidae
.	Genus *Actinomonas*
Class Slopalinatea	Genus *Pteridomonas*
Order Slopalinida	Order Ciliophryida
Family Proteromonidae	Family Ciliophryidae
Family Opalinidae	Genus *Ciliophrys*
Class Eustigmatophyceae	Order Actinophryida
.	Family Actinophryidae
Class Actinomonadea	Genus *Actinophrys*
.	Genus *Actinosphaerium*

The solution is not free of problems. Should there be a level at which the codes and classification become coherent? For example, should each family and its component subtaxa be treated under the jurisdiction of one code. As the ICZN and ICBN apply fully up to the family level, this restriction might reduce the number of problems. The cost would be a loss of some flexibility. This solution does not eliminate the difficulties of authorities who cannot agree on which code to apply. It would still be possible to treat the causative agents of sleeping sickness as plants. The solution may reduce this problem if workers are guided by the tradition of nomenclatural practice at the genus and species level. These proposals do not eliminate the problem of homonyms and synonyms. Where these do occur, the most appropriate name can be identified on a case-by-case basis, as noted above. This might be done by adopting the most stringent conditions (for a name to be used it must be acceptable under both codes), or by a more lenient method of "fine tuning" (for a name to be used it must be valid under one code, and the meaning of homonyms may be made clear by context). In order to recognize this means of resolving problems and to facilitate its adoption, the ICZN and ICBN would need to be amended, especially in respect of the statements of exclusivity and in their rules governing the transfer of taxa.

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THE DEVELOPMENT OF METHODOLOGY FROM THE GELEI-HORVÁTH SILVER IMPREGNATION METHOD TO SCANNING ELECTRON MICROSCOPY

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On the occasion of the First International Conference of Hungary on Protozoology, it is appropriate for protozoologists to gather here in Budapest to pay homage to ore of the great protozoologists of the twentieth century, Professor József (von) Gelei. As was pointed out by Maucha in a "Gedenkschrift" to Professor Gelei in 1954 (1), Professor Gelei was a biologist, ecologist, cytologist, geneticist, histologist, and limmnologist, among his other attributes. We need only to peruse his list of publications (2) to amplify this assessment of his versatility and breadth. To ciliatologists, he is, of course, remembered for his meticulous use of cytological techniques and his critical analysis of the structures revealed by these techniques, particularly the application of the Gelei-Horváth method, which was published in 1931 (3).

As is often the case in science, two completely unrelated fields of endeavor suddenly come together and open a whole new area of study, thus is the case of scanning electron microscopy (SEM) and its use in protozoology. József Gelei began publishing in the mid-twenties on the various uses of fixatives and stains in combination (i.e. osmium-toluidine blue, osmium-formalin, sublimate-formol with gold or silver nitrate or both). The object of his endeavors was to preserve as best as possible the "natural" condition of the protozoa he studied. This, as Parducz (4) has pointed out, led to the development of instantaneous fixation methods. In 1935, while the Geleis were refining the silver and gold staining techniques, Max Knoll in Germany hypothesized that an electron optical instrument could be built which used a stream of electrons that could be scanned across a specimen and the electrical signal caused by this interaction captured. After years of development, the first commercial

scanning electron microscope was produced in 1965 (for a historical review of the development of SEM, see Oatley et al., 5).

With the development of the instantaneous fixation techniques and the availability of SEMS in the late sixties, protozoologists had at their disposal the potential to examine the delicate surface features of the protozoa at high magnification with excellent spatial resolution ($\leq$ 200Å). The major obstacle remaining before protozoa could be examined in the vacuum environment of the SEM was adequate drying techniques. Drying specimens in the air, after dehydration from organic solvents or from an aqueous environment, creates excessive surface tension, resulting in drying artifacts (6). Horridge and Tamm (7) combined instantaneous fixation with the Anderson critical point method of drying (8). These authors demonstrated the complex ciliary beating patterns as seen in Opalina. At about the same time, Marszalek and Small (9) demonstrated the use of freeze-drying techniques as a means to overcome drying artifacts. Thus in the late sixties, the basic techniques were developed and protozoologists took full advantage of this technology as illustrated in the voluminous literature that has appeared over the last two decades incorporating SEM. For example, the recently published "Illustrated Guide to the Protozoa" has well over 100 scanning electron micrographs of representative species from most of the major taxonomic groups of protozoa (10). With this brief background, I would like to review with you some of the applications of SEM in our laboratory and, if I may, inquire into the development and application of SEM in the future.

The well-defined stages of oral regeneration in Stentor coeruleus using SEM and TEM were studied (11, 12). In figure 1, the intact morphostatic oral area including the membranellar band (MB), frontal field (FF), and orifice of the buccal cavity (BC) are clearly seen. Key morphogenetic events that occur during regeneration are the sprouting of cilia in the oral primordium, figure 2, and the dedifferentiation of remnants of the old buccal structures (i.e. MB and FF) which commences approximately 4 hours after regeneration has started (figure 3). Although these two stages have been studied extensively by Pelvat and de Haller (13) and Bernard and Bohatier (14), it is still not clear to what extent the old frontal field is incorporated into the new frontal field. Figure 4 shows the adult form of the marine suctorian ciliate Ephelota. Figures 5 and 6 are of a sapropelic heterotrich ciliate, probably a species of Metopus.

In these micrographs the metachronal wave patterns of the somatic cilia and the undulating membrane are seen. The predaceous, carnivorous ciliate Dileptus has been fixed during the early phases of the capture and ingestion of Tetrahymena (figures 7-8). The material covering the ventral surface of the proboscis and portions of Tetrahymena is more than likely toxicyst residue. Inspection of representative SEMS of the parasitic flagellates Giardia (figure 9), Phytomonas (figure 10), and Trypanosoma equiperdum (figure 11) reveals the structural details of these economically important organisms. Figure 9 illustrates the use of the dual magnification split screen capability of some modern SEMS. The viewer can examine portions of the same field of view at low and high magnification, simultaneously, isolating structural details. Trypanosoma equiperdum (figure 11) was fixed in division as indicated by the two flagella. A microneme is visible on the posterior end of the cell (arrow).

To this point, we have explored the use of secondary electrons, those electrons ejected from the specimen atoms close to the surface by the primary beam electrons. Additionally, some of the high energy primary beam electrons are elastically scattered from the specimen, retaining 80-90% of the beam energy, and with special detectors can be collected as "backscatter electrons" (BSE) and imaged. Elements of high atomic number (e.g. gold, silver, etc.) will produce images of high contrast with a spatial resolution < 100nm (15). Small et al. (16) have demonstrated the use of BSE from protargol impregnated specimens (Bodian method) and Tellez et al. (17) have used the Fernandez Galiano silver impregnation method, although according to these authors, this method does not "stain" microtubules.

Using the protargol techniques of Tuffrau (18) and Ng and Nelsen (19) on Stentor and Tetrahymena respectively, we have been able to image microtubular complexes in the BSE mode of the SEM. Figure 12 is a BSE image of the posterior end of Stentor. Individual kinetosomes and microtubular complexes (i.e. ciliary axonemes, post ciliary microtubules) are resolved in the kineties of the somatic and frontal field ciliature of Stentor. Figures 13-16 compare the secondary and backscatter images of the kineties. The dikinetids of the somatic kineties are clearly seen linked to adjacent dikinetids by microtubules. A portion of the oral apparatus of Tetrahymena can be compared in secondary and backscatter images (figures 17-18) providing 3-dimensional topological information

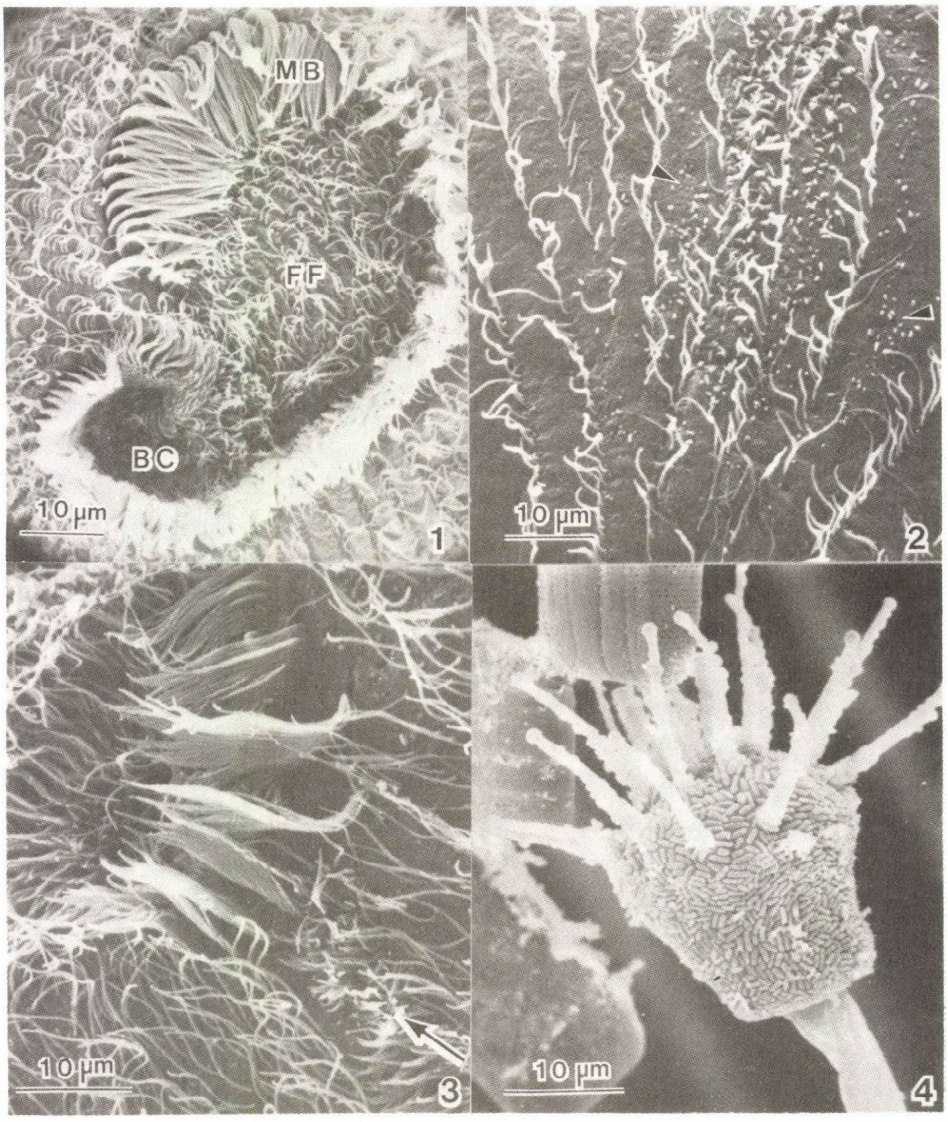

Fig. 1. Anterior pole of Stentor coeruleus, the membranellar band (MB), frontal field (FF) and buccal cavity (BC) are indicated.

Fig. 2. Portion of the ventral surface of S. coeruleus, the "sprouting" cilia (arrows) of the oral primordium are seen between several somatic kineties, cell fixed 1½ hrs. after regeneration was initiated.

Fig. 3. Late stage of MB regeneration, the anterior portion of the new MB (arrow) has come to lie near the dedifferentiating "old" MB.

Fig. 4. The marine suctorian ciliate Ephelota covered with rod-shaped bacteria. Organisms obtained from Sapelo Island, Georgia, USA, by Dr. William Henk.

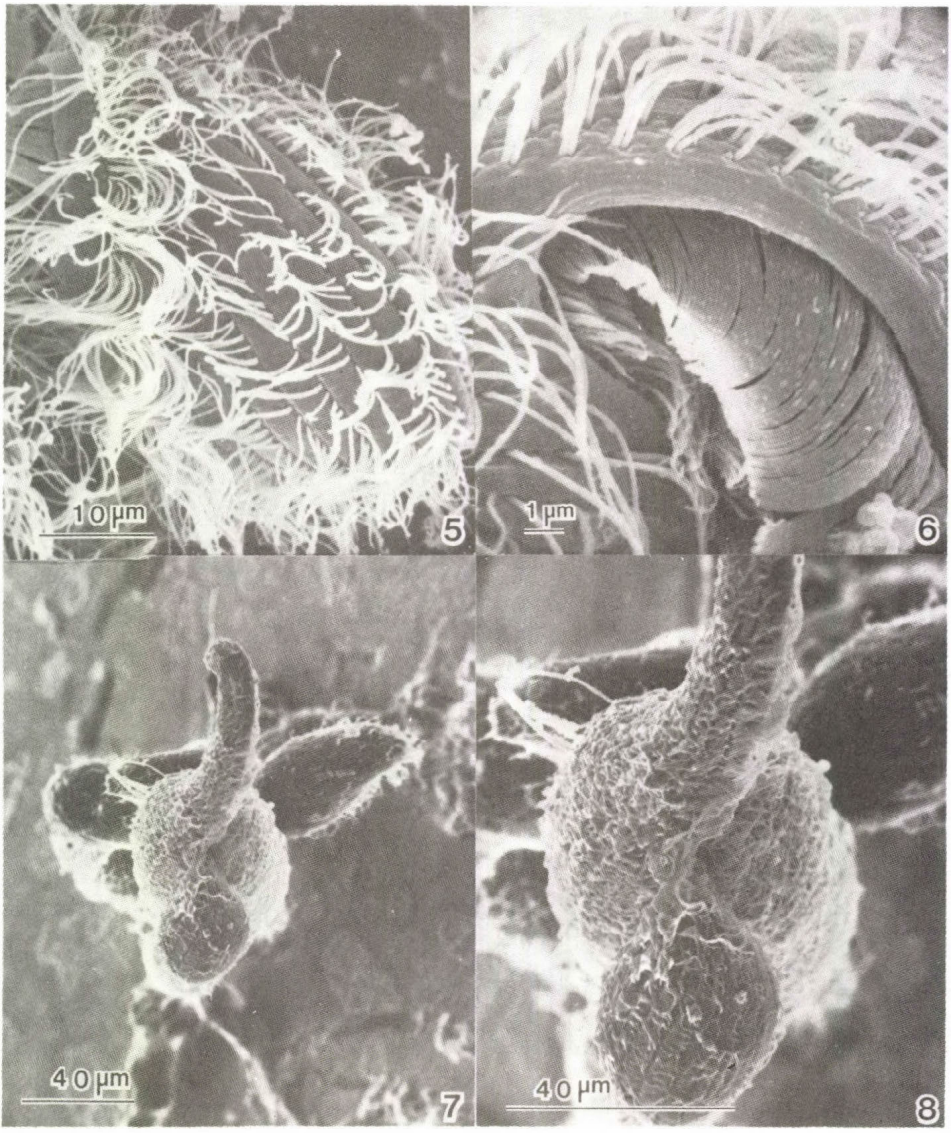

Fig. 5. A heterotrich ciliate, probably a species of Metopus, fixed from a pond sample. The metachronal waves of beating cilia were preserved by the instantaneous fixation method of Parducz. Notice the smooth surface of the cell.

Fig. 6. Higher magnification of the undulating membrane and adjacent dikinetids of the ciliate shown in Fig. 5.

Figs. 7-8. Two different magnifications of Dileptus feeding on Tetrahymena, the prey is about to be swallowed. Notice in particular the material along the ventral surface of the proboscis and enveloping a portion of Tetrahymena. The cytostome of Dileptus is indicated with an arrow.

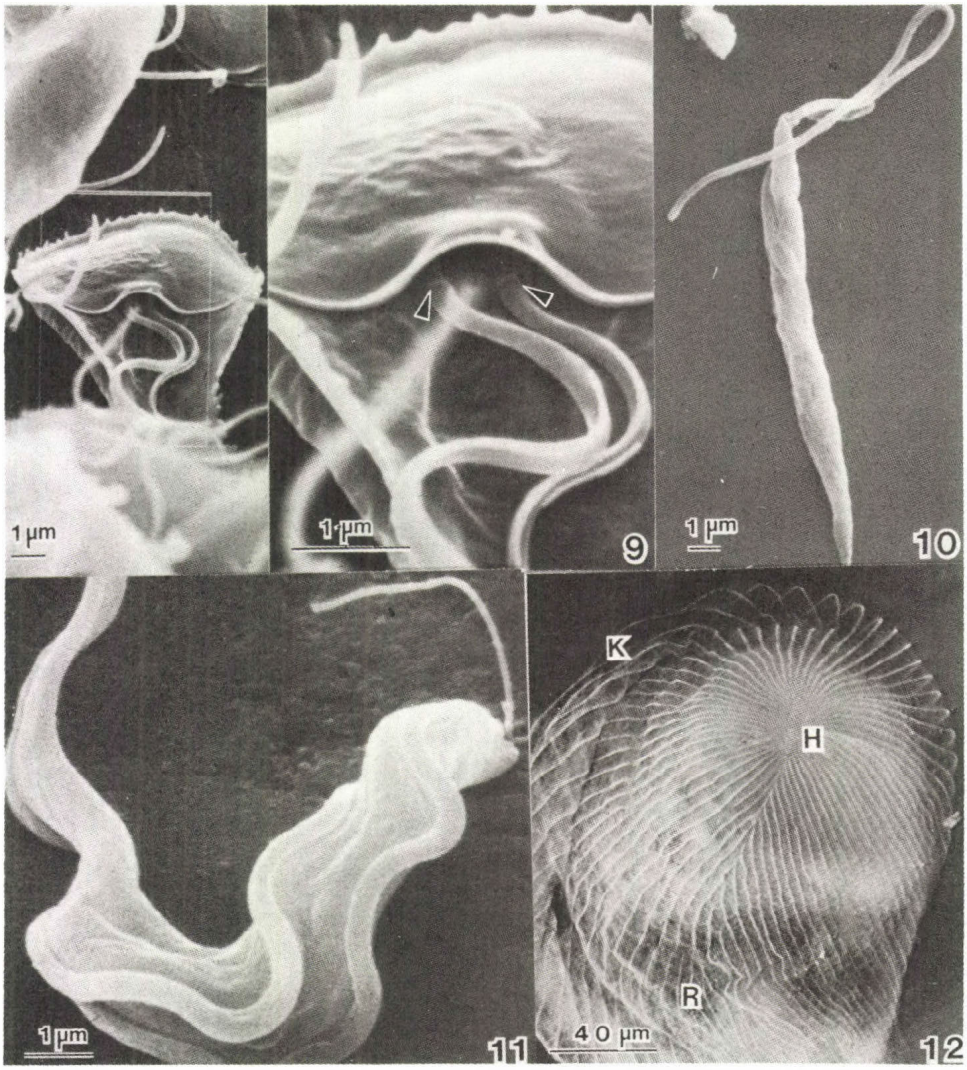

Fig. 9. Ventral surface of Giardia intestinalis seen in split screen dual magnification. The portion of the specimen outlined by the box is projected on the right. Notice the annuli (arrows) on the ventral flagella below the attachment disk. Giardia kindly provided by Dr. Govinda Visvesvara, Centers for Disease Control, Atlanta, Georgia, USA.

Fig. 10. Phytomonas sp. The flagellum is longer than the cell.

Fig. 11. A dividing Trypanosoma equiperdum depicting 2 flagella and the micronemes found on the posterior end of these cells.

Fig. 12. Backscatter Electron image of the posterior pole of Stentor coeruleus prepared using the protargol technique of Tuffrau. The cells were dried by the critical point method directly on albuminized coverslips. The argentophilic kineties (K) hold fast (H) and ventral ramifying zone (R) are seen.

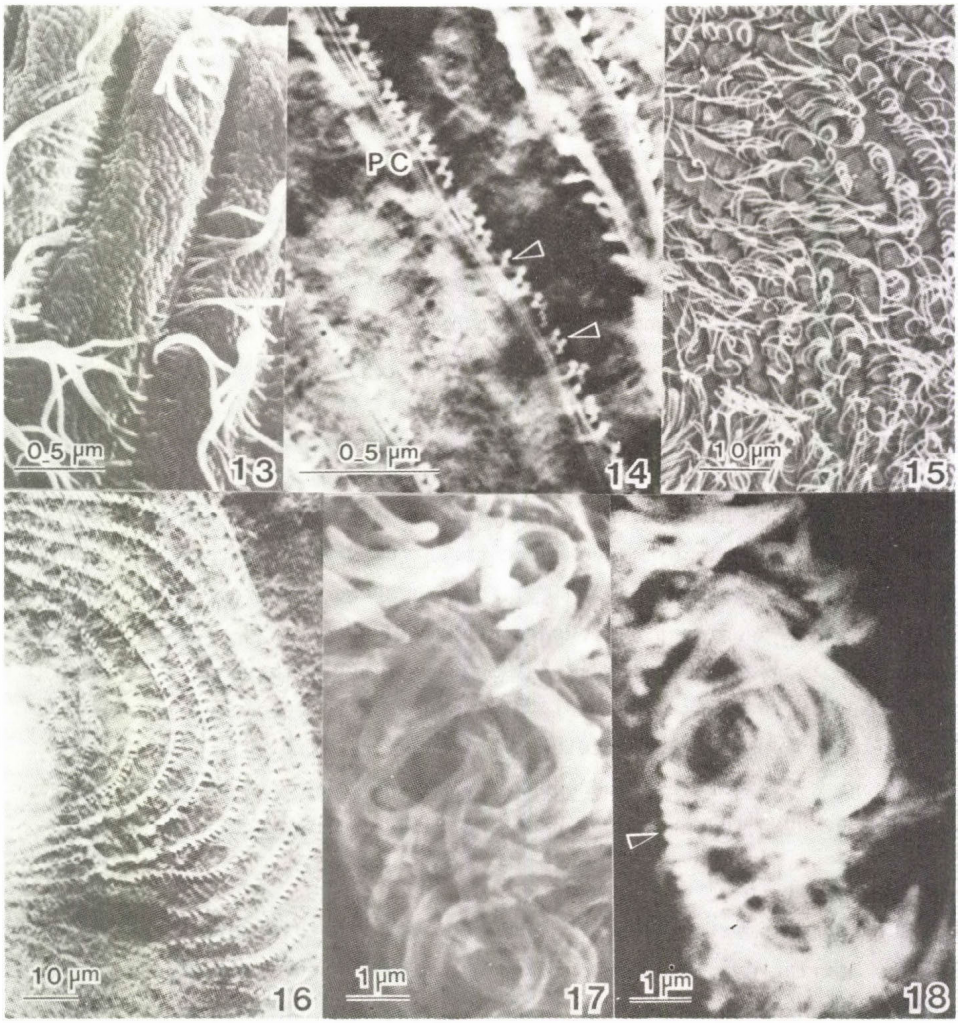

Fig. 13. Secondary electron image of the cortical surface of Stentor, cobble-stone appearance is due to pigment granules below the surface of the cell between kineties.

Fig. 14. BSE of kineties, the dikinetids (arrows) and postciliary microtubules (PC) are seen (carbon coated only).

Fig. 15. Secondary electron image of frontal field.

Fig. 16. BSE image of protargol stained (Tuffrau) FF, corresponding to Fig. 15 (carbon coated).

Fig. 17. Secondary electron image of the oral area of Tetrahymena, protargol stained (Ng-Nelsen), carbon coated.

Fig. 18. BSE of same oral area as Fig. 17, kinetosomes of the UM are indicated.

with compositional information (i.e. atomic number contrast). Switching between the two detectors allows for examination of surface and subsurface features, so important in morphogenetic studies. These imaging techniques bridge the gap between light and TEM and have great potential in cell biology (20).

Specimens viewed in the BSE mode can also be analyzed simultaneously by energy dispersive x-ray analysis (EDS) to confirm elemental composition. We have been using EDS to supplement BSE analysis and to identify inclusion bodies in trypanosomes (polyphosphate vesicles, 21). However, the preservation of material for x-ray microanalysis is critical and not always comparable with morphological studies, since most fixation procedures and/or dehydrating and embedding techniques cause a redistribution or loss of ions (see especially Morgan, 22).

Like SEM, the high voltage electron microscope (HVEM) can provide three-dimensional information about the structure of protozoa. The high accelerating potential (> 750Kev) increases the penetration of electrons through thick specimens while retaining good spatial resolution. We have used HVEM to produce 3-dimensional models of the mitochondria of several species of trypanosomatids (23). The models were obtained by collating photomicrographs of serial thick sections ($\frac{1}{4}$-$\frac{1}{2}$ µm thick). The complex mastigont system of Tritrichomonas foetus and other trichomonads was also studied by HVEM, using stereo-pair analysis of thick sections (24).

Epilogue

The progress we have made in understanding the biology of the protozoa can be attributed to the foundations provided by the innovations of eminent scientists such as József Gelei. His insights on the use of staining and fixation techniques provided generations of microscopists with the methodology to study protozoa in near nature conditions. The SEM has gained its rightful place in protozoan research, particularly when used in combination with TEM and light microscopy. Modern SEMS have resolutions approaching 35Å. Consequently, structures heretofore only seen in the TEM will be subject to analysis in the SEM.

The future of electron microscopy lies in the development of preparative techniques to utilize and maximize the total analytical capabilities of electron optical instruments. The use of BSE, EDS and secondary electron imaging in consort opens new avenues of research, promulgating analytical methods of analysis. These are exciting times and in this

writer's opinion, we are in the "Alpha" stage of electron optical methodology.

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PROGRESS IN METHODOLOGY IN THE LAST FIVE DECADES

(Abstract)

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Research in protozoology started with the development of the light microscope in the seventeenth century. Since the cell theory was proposed in 1838, the protozoan organization was considered to be a single cell. In protozoology and cell biology an enormous variety of experimental techniques have been developed to study cells. Most advances of the last 5 decades including the exciting ones of recent years depend on the introduction of new methods. The limit of resolution by the wavelength of visible light can be reduced by using electrons instead of light. Thus, the electron microscope resolves the fine structure of the protozoan cell and three-dimensional images can be obtained. Furthermore, individual macromolecules can be resolved by electron microscopy. Many other new methods belong in the field of cytochemistry, physiology, chromatography, electrophoresis, studies of fractionated cell components; cell motility, separation of organelles, reproduction of DNA sequences by cloning and many other methods.

*This was the last paper by Prof. Scholtyseck. He died before the beginning of the Conference.

Symposia Biologica Hungarica 33 (1986)

PROGRESS IN METHODOLOGY DURING THE LAST FIVE DECADES

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This presentation is to honor not only József Gelei, to whom the entire Conference is dedicated, but also the recently deceased Professor Erich O. Scholtyseck, an eminent protozoologist and my personal friend, who was asked originally to deliver this lecture.

Admittedly, it is a difficult task to review in 20 minutes the progress in methodology employed in studies of protozoa during the past 50 years. Since over two-thirds of this period coincided with that of my scientific career, I had the opportunity to follow much of that progress.

I wish to emphasize that, because of time limitations, I shall mention only very briefly some of the important methods and even shall have to omit a few from consideration. Among the last will be the monoclonal antibody procedures, now very widely used, and the recombinant DNA technology whose significance and potential are enormous.

Clearly, the development of methods for studies of protozoa has in a large measure paralleled that of techniques applicable to cells in general.

Were I to use ciliates, the organisms investigated extensively by Gelei, to illustrate my presentation, I would have to depend mainly in work done by others. However, since one feels most comfortable with the material with which one is most familiar, I shall employ primarily trichomonads to illustrate the results obtainable by various methods.

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* Acknowledgements of grant support are to be found in the publications cited in the list of references.

Light Microscopy

Before introduction of phase and interference microscopy, studies of living material utilized first axial illumination and later also dark field. A diagram of the intestinal trichomonad of man based on dark-field observations was published by Kirby (1943). It reveals the arrangement of the flagella which was difficult to resolve in fixed and stained preparations available in the mid 1940s. A drawing of another flagellate, Retortamonas, seen in dark-field, can be found in one of his later papers (Kirby and Honigberg, 1950). Although the first phase-contrast microscope was designed by Zernicke in 1932, its wide application to studies of protozoa came considerably later; in California, we did not employ phase-contrast microscopy until the mid 1940s. When used properly, this technique reveals in living organisms nearly all the structural details seen in fixed and stained preparations. This is evident from the micrographs and diagrams of Trichomonas vaginalis included in the report of Honigberg and King (1964) -- here one can compare the results obtainable with living cells by phase-contrast microscopy and with fixed and stained flagellates by bright-field microscopy.

When the more advanced methods for observations of living protozoa were still in early stages of development, structural details of these organisms could be studied in fixed and stained preparations (Corliss, 1973). The iron hematoxylin method as devised originally by Heidenhain (1892) or one of its modifications was among those employed most widely. A variety of fixatives have been used for materials to be stained with the aid of this technique; among them, picric acid-containing solutions, e.g. Bouin's fluid, and chromic acid-, and OsO_4-containing fluids, e.g. Flemming's, Champy's, have been found very satisfactory for a number of protozoan species. Examples of T. vaginalis preparations stained with iron hematoxylin are shown in the photomicrographs of this species and in composite diagrams based on examinations of many organisms (see Honigberg and King, 1964).

Just as the knowledge of infraciliature has been advanced by the application of various silver-impregnation methods (Corliss, 1953, 1963, 1973), used extensively by Gelei, our understanding of the structure of

flagellates has been aided greatly by the introduction of strong protein silver (protargol) staining technique (for the history and details of this method, see Honigberg, 1973). The photomicrographs included in the paper by Honigberg and King (1964) illustrate the details revealed by protargol staining, which is useful also for studies of ciliates (Corliss, 1973; Honigberg, 1973; Jerka-Dziadosz and Frankel, 1969).

In 1952, Nomarski patented the system of differential interference contrast. This system, which bears the inventor's name, is especially useful in studies of the surface and of structures located on the surface of living organisms; some internal structures can also be demonstrated with its aid. Furthermore, differential interference contrast can be employed, often with dramatic results, for fixed and stained protozoa. Fair examples of the results obtainable by the Nomarski method can be seen in the photomicrograph of a dividing Balantidium coli (Zaman 1978) and in one of Trypanosoma brucei (Mendez and Honigberg, 1972).

All the foregoing methods, in combination with cytochemical techniques (see, for example, Mellors, 1955, Pearse, 1961), which I shall not discuss, have virtually exhausted the opportunities presented by light microscopy for studies of protozoa.

Electron Microscopy

The advent of electron microscopy allowed the extension by about 100 times of the limits of resolution achievable by the most effective employment of the light microscope. Although the first commercial transmission electron microscope (TEM) was produced by Siemens in 1939, many years passed, in part because of World War II, before this instrument was perfected to the point at which it could become a routinely used tool. Furthermore, the protozoa, many of which are difficult to fix and to section, could not be studied properly before the introduction (in 1963) of superior fixation methods, i.e. glutaraldehyde, usually followed by OsO_4, and of embedding media, e.g. Epon and Araldite (in 1956). Furthermore, acceptable sections of some protozoa, e.g. many flagellates, can be obtained only with the aid of diamond knives.

The impact of TEM on our understanding of structural details and phylogenetic relationships of various protozoan groups has been or will be discussed during this Conference. As in the previous instances, I shall

employ trichomonads to illustrate the advances made through the use of TEM. There are two subdivisions of trichomonads equipped with an undulating membrane and a costa, an organelle that supports the membrane. A typical representative of one of these subdivisions (subfamilies) is Trichomonas, and, of the second, Tritrichomonas. Fine-structural similarities and differences between these two genera can be seen by comparing the composite line diagrams included in the papers published by Mattern et al. (1967) and Honigberg et al. (1971). Important differences exist between many organelles, e.g. undulating membranes, costas, or the peltar-axostylar complexes, found in the two genera. It should be noted, however, that these organisms share also many characteristics and that hydrogenosomes, organelles of great importance in metabolism of trichomonads (they employ protons as terminal electron acceptors and produce molecular hydrogen), are identical in their fine structure and distribution in these two organisms; this actually is true of all members of the order Trichomonadida. Electronmicrographs of Trichomonas and Tritrichomonas, included in the aforementioned reports, illustrate the actual appearance of the structures shown in the line diagrams.

Scanning electron microscopy (SEM) has also been found useful in studies of protozoa. This is a more recent technique, the first commercial scanning unit having become available only about 20 years ago. The SEM, which reveals structure of cell surface and of organelles found on the surface, produces three-dimensional images. Although capable of much lower resolution than TEM, SEM is helpful in examination of complete organelles at useful magnifications much higher than those utilizable in light microscopy. The results that can be obtained with the aid of SEM are shown in the micrographs of Trichomonas and Tritrichomonas published by Warton and Honigberg (1979). In these micrographs distinct differences can be noted between the undulating membranes of the two genera. Also, one sees the details of the periflagellar canals whose walls are supported by the peltas. There are other means of obtaining three-dimensional views of various structures, e.g. of groups of kinetosomes, in relatively thick sections (up to 1 μm). In these instances, the specimens are tilted in the electron beam and are photographed from two different angles. When the pairs of electronmicrographs are examined with stereoglasses, a three-dimensional image is created. The use of thick specimens is possible only in high-voltage electron microscopes in which the electron beam,

accelerated through one million or more volts, is capable of penetrating thick sections.

Finally, membranes, their interior, intramembranous particles, and organelles made up of membranes can be studied by the freeze-fracture techniques, developed between 1957 and 1966. In freeze-fracture preparations, the fracture plane often passes through the hydrophobic middle areas of lipid bilayers; thus, the interior of the membranes is exposed. The fracture planes are coated with platinum, and all organic material is dissolved. The floated replicas, examined in a conventional transmission electron microscope, are seen to contain intramembranous particles which represent the large protein molecules that pass through the lipid bilayers. Such particles are shown in cell membranes of trichomonads pictured in one of our recent reports (Honigberg et al., 1984). In addition, the micrographs show, among other details, the intricate internal structure of the undulating membrane in Trichomonas, the orderly arrangement of intramembranous particles marking the junctions between the recurrent flagellum and the margin of the undulating membrane, the rosette-like accumulations of particles on the surfaces of the anterior flagella (such rosettes are found on the anterior flagella only in trichomonads and hypermastigotes, providing additional evidence for the close relationship of the two groups), the structure of hydrogenosomes, and the details of the Golgi complex.

CULTIVATION

Time does not permit a discussion here of the important role played by the development of methods for axenic cultivation, especially in chemically defined media, in the studies of nutrition and biochemistry of protozoa. Information on cultivation methods may be found in several multiauthored books (e.g. Hutner and Trager, 1953; Jensen, 1983; Taylor and Baker, 1978) and in works dealing either with groups of genera and species or with individual genera and species; for example, chemically semidefined or defined media have been developed recently for trichomonads (Lindstead, 1983; Wang et al., 1984).

Fractionation

After the arrangement of cell organelles and of large macromolecular assemblages have been determined by various microscopic methods, further understanding of cells, and thus of protozoa, has required the employment of biochemical methods. Although many such methods were available in Gelei's time, especially during the later part of his career, only relatively few investigators applied them to studies of protozoa.

Biochemical analysis requires disruption of cells in such a way that various cell components, i.e. nuclei, mitochondria, lysosomes, peroxisomes, or hydrogenosomes, can be separated, typically by centrifugation, with their activities intact. Different kinds of centrifugation at various speeds are being employed, e.g. continuous or discontinuous gradient centrifugation. Speeds that produce forces as high as 150,000 g can now be used, the highest being needed for separation of very small molecules. With regard to protozoa, and especially trichomonads, a good example of the results obtained from fractionation by centrifugation can be found in the reports from the laboratory of Müller (e.g. Müller, 1973; Lindmark and Müller, 1973) which describe the localization of a number of enzymes, crucial in metabolism of Tritrichomonas foetus, in a fraction consisting of microbody-like organelles, each delimited by a double membrane, the hydrogenosomes. (These organelles correspond to the paraxostylar and paracostal granules of the light microscopists.) The appearance of the large particle or hydrogenosomal fraction can be seen in one of Müller's (1973) electronmicrographs. By isopyknic centrifugation in a sucrose gradient of the hydrogenosomal fraction obtained with the aid of differential centrifugation, it was demonstrated that several enzymes were localized in a band around a density of 1.24. These enzymes, the localization of some of which is shown in figures published in Müller's (1973) report, include pyruvate:ferredoxin oxidoreductase, hydrogenase acetate thiokinase, acetyl-CoA transferase, and superoxide dismutase, in addition to malate dehydrogenase (decarboxylating), which is a marker for hydrogenosomes.

Chromatography and Electrophoresis

A major aim of cell biology, including biology of protozoa, is to obtain cell-free systems capable of carrying out complex life processes. Analysis of cell-free systems depends upon separation of individual cell components of these systems. Various kinds of chromatography have been used for fractionation of sugars, amino acids, and of entire proteins. Thin layer chromatography is typically employed for smaller molecules, while column chromatography is preferred for large molecules, e.g. proteins. One of the most efficient procedures is affinity chromatography in which antibodies for specific proteins are employed for their purification. Although used routinely in biochemical and immunological analyses of protozoa, the chromatographic methods will not be discussed here. (Many papers published in journals such as J. Protozool., J. Parasitol., Exp. Parasitol., or Biochem. Molec. Parasitol. deal with application of various chromatographic techniques to protozoa.)

On the other hand, I wish to mention briefly the electrophoretic methods, based upon charge differences of the groups of amino acids located on protein surfaces. Among these methods, the sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), developed in the middle 1960s, has been found to be the most effective for protein analysis. This technique which separates polypeptides according to size, aids also in estimating the molecular weight and provides information about the subunit composition of a protein. We have used SDS-PAGE extensively in studies of biochemical and immunologic properties of trichomonads. Many figures of such gels are included in our reports which are in various stages of preparation. Protein composition of trichomonads, e.g. Trichomonas vaginalis, can be seen in a photograph of silver-stained gels which are shown side by side with a similarly stained gel containing proteins of known molecular weights used as standards.

I hope that this brief overview of the progress in methodology employed in studies of protozoa has provided some insight into what has been happening during the past five decades. Needless to say, we have come a long way from the time when protozoa were examined exclusively with the aid of the light microscope. Admittedly, however, Gelei and his predecessors and comtemporaries who employed light microscopy learned a great deal about these small and complex organisms. At times one wishes

that present-day investigators looked at the cells and had a chance to admire their beauty before solubilizing them.

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THE CILIATE CORTEX STUDIED BY TEM AND SEM CRYOFRACTURE TECHNIQUES*

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In the recent decades there has hardly been a technique in protistology that has had a greater impact than electron microscopy and while thin sectioning is certainly the most efficient approach to fine structural investigations those techniques which omit any chemical treatment have led to new unexpected observations, moreover they serve as necessary controls for routine EM techniques. Here, we would like to report on the particular usefulness of TEM and SEM cryofracture techniques in studying the cortical organization of ciliates. It is the structural diversity of both the somatic and buccal cortex which in addition to the overall cell shape determine the most characteristic features of the ciliates. Apart from the microtubular and microfibrillar entities of the infraciliature, the cortical membrane systems in form of the pellicular alveoli and the membrane-bound organelles are of utmost significance for species identification and studies on morphogenesis and phylogeny in these most complex unicellular eukaryotes.

Freeze-fracture (FF) technique introduced by Steere and developed to technical perfection by Moor (1971) has literally opened a new dimension of membrane biology. This technique allows to study the distribution of integral membrane proteins

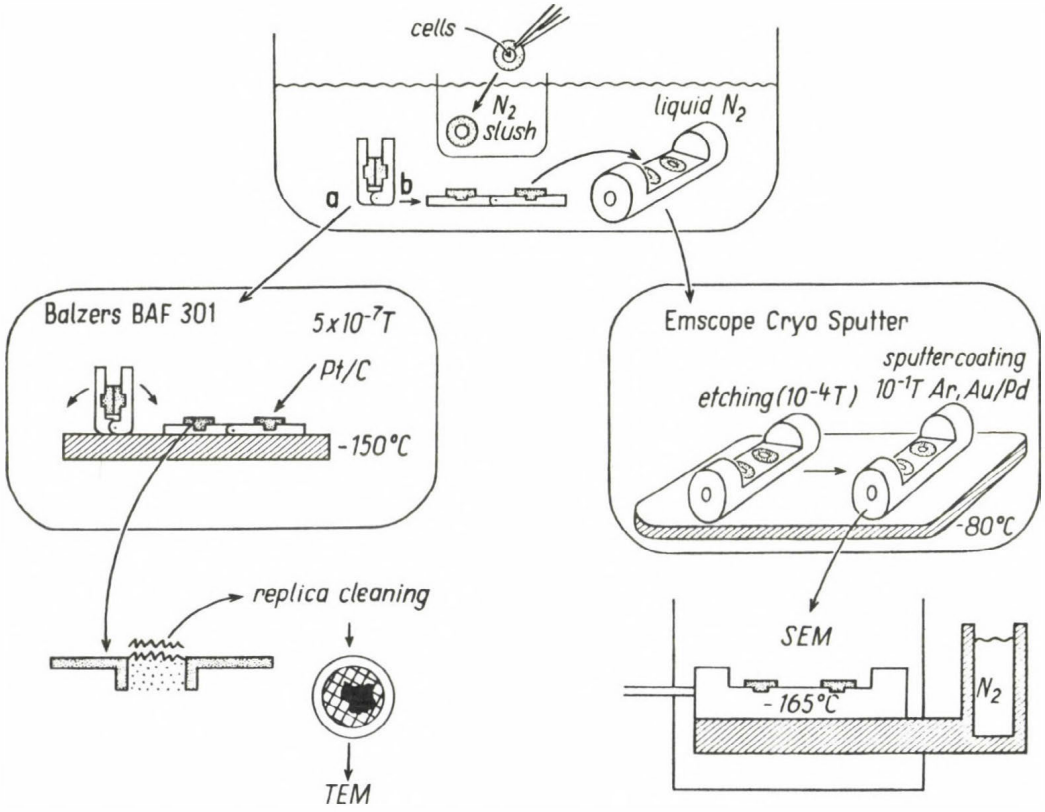
*Dedicated to the memory of Jőzsef Gelei whose ingenious staining techniques were almost forgotten in the age of electron microscopy.

and has had a crucial impact on the currently most popular concept of biological membranes, the "fluid mosaic model" of Singer and Nicolson (1972). FF technique combines the high resolution of TEM with a three-dimensional view of the structure studied and it is this latter aspect which is of particular interest for ciliatologists. Before we list the various advantages of this type of preparation it seems appropriate to describe briefly the main steps of our routine procedure.

Freeze-Fracture Technique for Transmission Electron Microscopy

Living cells (or cells fixed in glutaraldehyde and infiltrated with 30% glycerol after thorough washing) are placed into a pair of double replica rivets from Balzers. The specimen is then dipped as quick as possible into nitrogen slush and transferred to a Balzers double fracture device submersed in liquid nitrogen. The double fracture device is then put on the cold stage of a Balzers machine BAF 301, evacuated to $5 \cdot 10^{-7}$ Torr, fractured at $-150^{\circ}C$ and omitting the etching step it is immediately shadowed with platinum and carbon using electron beam evaporation sources. The cleaned replicas are mounted on uncoated sticky grids and viewed in a TEM. While the FF technique was originally designed to study unfixed cells it should be stressed that numerous experiments with both living and fixed ciliates have shown that all static morphological features described in the following are not impaired by the use of fixed cells. The benefit of using fixed cells is the higher yield of larger replicas since extrusomes of unfixed cells will often tear the replica into pieces due to the sudden hydration of the extrusomes as soon as the specimen with the replica is put into an aqueous medium.

1) FF technique is particularly useful to study the shape and distribution of the pellicular alveoli. There is a very good correlation between Chatton-Lwoff preparations and the FF picture supporting the conclusion that one of the main sites of silver deposition are the border-lines of the pellicular alveoli. The higher resolution of the FF image is particularly



Comparison of TEM freeze-fracture technique (a) with SEM cryofracture technique (b).

helpful in ciliates with small and flat pellicular alveoli as in certain karyorelictine, heterotrichid or oxytrichid ciliates where its existence is even questioned at times.

2) No other technique allows such a detailed study of the often complex cytotopography at the level of the plasma membrane. This is particularly evident if one compares the FF picture with the results of the thin-sectioning or the routine SEM technique. Thin sections cut across membranes according to their arbitrary orientation to the cutting plane of the embedded specimen and laborious reconstruction from serial sections is needed to get a three-dimensional view of a complex surface. With routine SEM technique using Parducz fixation and critical point drying, a technique which beyond studying the living cell gives the most "vivid" picture of a ciliate, the proper cell surface is often hidden by a dense pelt of cilia. In FF, however, the fracture line may follow the hydrophobic interface of the plasma membrane over a large area as recently shown for small ciliates like Cyclidium (Bardele, 1983a). A detailed knowledge of the various landmarks in the ciliate cortex, e.g. suture lines, oral structures of various kinds, excretory porus and cytoproct as well as the precise distribution of somatic monokinetids and dikinetids are of utmost importance for morphogenetic studies. While these features normally are studied by silver impregnation techniques (Chatton-Lwoff , Protargol or Fernandez-Galiano) it is evident that for certain structures, e.g. the parasomal sacs and the individual cilia in compound ciliary organelles these techniques have their limitations.

3) As soon as we get interested in the macromolecular arrays of the intramembraneous particles we discover a fascinating diversity of precisely ordered membrane domains in the ciliary membrane, the plasma membrane, the alveolar membranes and the membrane bounding the extrusomes. While the functional significance of these membrane domains is still largely unknown we have just begun to unravel their morphogenetic and phylogenetic implications.

a) There are in the first place the various particle arrays in the ciliary membrane, e.g. the double-stranded ciliary necklace (Gilula et al., 1972), the ciliary plaques (Wunderlich et al., 1972) in certain hymenostome and colpodid ciliates and other genus-specific arrays like the ciliary rosettes in Frontonia (Bardele, 1981). To date for none of these arrays a functional explanation has been given, but it is beyond doubt that these genetically fixed membrane characters are valuable traits for phylogenetic considerations. So far only one group of protists, the opalinids, has been found to have a double-stranded ciliary necklace as all the ciliates have (Bardele, 1983b). And it is certainly of particular interest that dicyemid mesozoans likewise have a double-stranded ciliary necklace contrary to all lower invertebrates which have a triple-stranded necklace (Bardele, unpublished observations). In addition to recent molecular data by Ohama et al. (1984) this may be taken as a further argument for a possible relationship between ciliates and mesozoans, thus weakening the view that the latter descended from flat worms by simplification due to their parasitic mode of life.

b) A functional prerequisite of all extrusomes in ciliates (trichocysts, mucocysts, pigmentocysts, toxicysts, haptocysts etc.) seems to be the attachment rosette which assembles in plasma membrane as soon as the extrusome approaches its docking site (Satir et. al., 1973). The attachment or fusion rosette is interpreted as a triggering device which under normal circumstances discharges its content only upon an appropriate stimulus from outside the cell. In addition to its receptor function the rosette seems to be involved in ion channelling during the early events of extrusome extrusion. But it was certainly premature to apply the concept of fusion rosettes to secretion in general. There is a strange difference in the number of particles in the attachment rosettes, while all "euciliates" have about 8 particles arranged in a ring with a central particle being present or not, all suctorians show 12 particles plus 1 in the haptocyst attachment rosette. The

double ring of particles around the fusion rosette in Paramecium is a special feature of this genus, it may help to find the precise docking site. Most non-hymenostome ciliates have a less regular patterning of their extrusomes. It is not yet known how they find their way to the docking sites which lie always between the border-lines of alveoli.

c) Other membrane domains such as the particle plates found in the plasma membrane in or close to the oral opening have also been interpreted as sensory fields (Allen, 1978a, b; Bardele, 1983a). It may also be noteworthy that all clavate cilia studied so far have a higher particle density compared with ordinary cilia. In certain cases these special cilia occur in organelles which like the paralabial organ of the ophryoscolescids have been suggested to serve sensory functions though the hard proof is still lacking (Bardele, 1981).

d) The position of those barren basal bodies which are lying immediately underneath the plasma membrane is easily detected by the so-called fairy rings of particles (Hufnagel, 1979). These structures will be helpful in studies of the turnover of cortical entities and the same probably holds for the two types of pellicular rugs found in precisely determined sites in the plasma membrane of Cyclidium (Bardele, 1983a). These domains which form the most complex particle arrays described so far pose intriguing problems at the level of macromolecular assembly.

The various highly ordered membrane domains found in the ciliate cortex seem to restrict the fruitful concept of membrane fluidity to the endomembranes and that part of the plasma membrane which covers the cytostome proper. The local remodelling of the cortical membranes during cell division and conjugation has remained largely unexplored. But since FF technique is a bulk-type preparation only precisely synchronized cultures will be suitable for studies of these dynamic events.

For a detailed illustration of most of the above mentioned membrane differentiations see Bardele, 1983a.

Freeze-Etch Scanning Electron Microscopy

For freeze-etch SEM we put living cells in a pair of Balzers double replica rivets, freeze them in nitrogen slush. The pair of rivets with the frozen cells is put into a Balzers double fracture device and fractured manually under liquid nitrogen. The 2 rivets with the frozen and now fractured cells are individually transferred to the specimen-holder of an Emscope Cryo Sputter. The specimen-holder is then transferred to the freezing chamber of the Emscope Cryo Sputter in order to attach it to the transfer unit. This is done under atmospheric pressure and while the nitrogen evaporates the chamber is fluted with argon. A shroud shielding the specimen is shut and the specimen-holder is retracted into the transfer unit, evacuated and kept under argon. The transfer unit is then docked to the precooled working chamber, where the specimen is etched for 10 min at $-80^{o}C$ in a vacuum of about $2 \cdot 10^{-2}$ Torr which yields an etching depth of roughly 10 µm. The still frozen probe is then sputter-coated with gold-paladium, taken back into the transfer unit and introduced onto a cold stage of a Cambridge Stereoscan 250 Mk II. The stage is kept at liquid nitrogen temperature and the specimen may be observed for more than 2 hours. It was found advantageous to operate the SEM with a lanthanum hexa-borid cathode which gives good resolution even at 3-5 KV and reduces the damage of the specimen which may be encountered with high KV.

Again, the advantage of this methods is the use of chemically untreated cells. The physical fixation by freezing is fairly rapid as monitored with Spirostomum. The highly contractile ciliate will contract only to about 3/4 of its body length compared to the contraction in any chemical fixative in which the cell will shorten to 2/5 of its length. Contraction of the cell seems to be combined with a spatial separation of the cortical cytoplasma comprising the pellicular ridges and layer of the myonemes from the rest of the cytoplasm. Such separation of ecto- and endoplasm is not observed when the cryofracture technique is applied. Only cells swimming in low ion strength

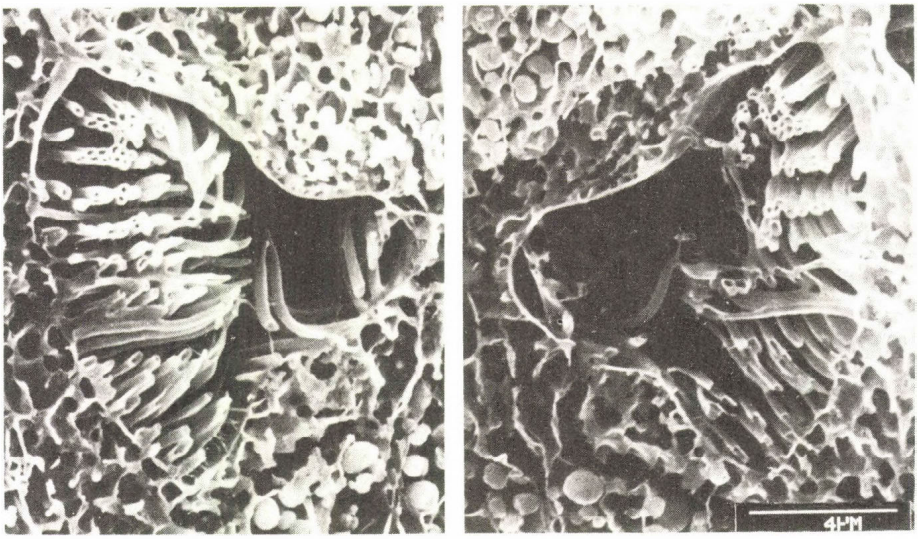


Fig. 1. Corresponding parts of an unfixed Spirostomum teres showing some of the individual cilia of the inner adoral membranelles.

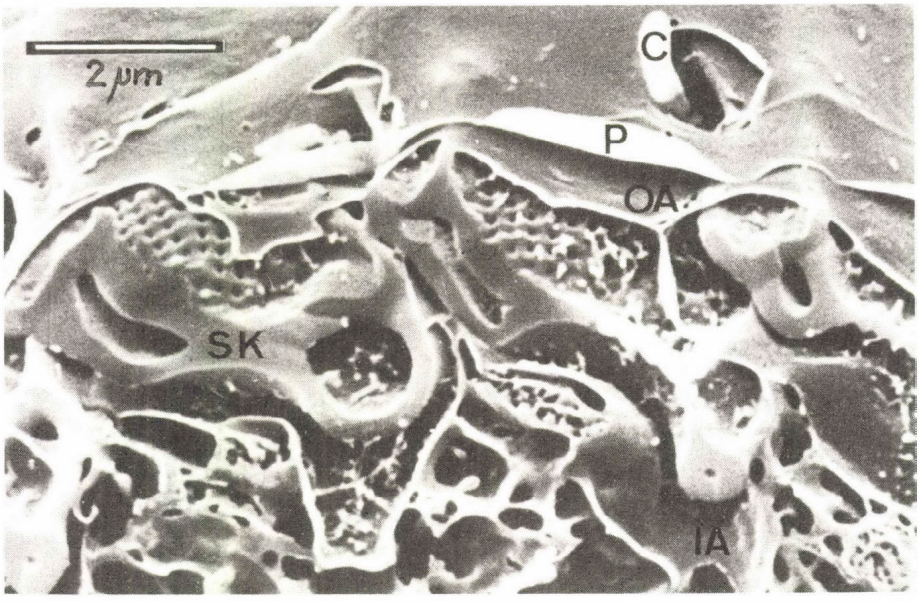


Fig. 2. Cortex of an unfixed Coleps amphacanthus with skeletal plates (SK) located between inner (IA) and outer alveolar membrane (OA). The OA is covered by the plasma membrane (P). Cilium (C).

media can be used for this technique. It is not applicable to marine ciliates because it is impossible to "etch" frozen seawater without artefacts (as beautiful as they may look).

Working at lower magnification it is fairly easy to find corresponding parts of a fractured cell as demonstrated in Fig. 1, which also illustrates the individual cilia. Their hollow aspect has to be regarded as an artefact of the etching procedure. On the other hand we may be close to the resolution limit of our SEM. A high resolution SEM technique for the observation of cytoplasmic structures has been described by Tanaka et al. (1984), but it uses chemically fixed tissue and though employing a field emission SEM it was so far impossible to demonstrate microtubules.

With our SEM cryofracture technique it is possible to look into hidden crevices and study e.g. the deeper regions of a cytostome. A particular promising example to demonstrate the usefulness of this technique is the work in progress by Huttenlauch (1985) on the cortex of Coleps amphacanthus where it is possible to observe the skeletal plates in situ (Fig. 2). Precisely timed studies on the morphogenesis of the skeletal plates have shown that the entire plate with its later massive rim grows from a grid-like precursor with a regular square pattern which is too delicate to be studied in thin sections. In principle, the SEM cryofracture technique can be combined with x-ray microanalysis, which would allow to study the early steps of skeletal plate morphogenesis at qualitative and quantitative level.

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DEVELOPMENT OF PROTOZOAN KARYOLOGY SINCE J. GELEI'S TIME

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The karyology of protozoans, and protists in general, certainly made enormous advances during the last decades, as did cell biology as a whole. It is however not quite easy to say what is actually "Gelei's time". József Gelei was active in protozoology, general zoology, and cell biology for nearly 50 years, from 1906 to his untimely passing in 1952. He was not especially working in karyology, but he has produced many outstanding contributions to the fine cytology of the infusorian cortex, due to his original method of silver impregnation (see, e.g., Gelei 1934). In the earlier years of his career, however, he has published several pioneering papers on chromosome pairing and meiosis in the Turbellaria. Therefore, and very arbitrarily, I shall limit myself to trace the major advances of protozoan karyology starting with the year 1950.

It must be said that by this time the protozoan nuclei were by far not an unexplored field. Many outstanding works made with the methods of light microscopy and cytochemistry were already available. One has only to recall the works of K. Bělař on mitosis and meiosis of Actinophrys, on nuclear division in Aggregata, as well as his summarizing critical book (Bělař 1926) on protozoan nuclei in general; those of E.Chatton on the nuclei of parasitic dinoflagellates; the studies of V.A.Dogiel on conjugation in Ophryoscolecidae; those of G.I.Poljansky on nuclear phenomena during conjugation in Bursaria; L.R.Cleveland's works on mitosis, meiosis, and sexual phenomena in Hypermastigida and Oxymonadida; K.G.Grell's papers on nuclear division in Stylocephalus and macronuclear

development in Ephelota; T.T.Chen's studies on the chromosomes in Opalinida and in Paramecium bursaria, and many others.

The rapid progress in protozoan nuclear cytology, marking the recent 35 years, is certainly due to the major developments in research technology. Among these is of course the use of electron microscopy, which resulted in the fact that now practically the entire protozoan morphology has been, so to say, translated into the fine structural language. Other breakthroughs are the development of autoradiography, especially at the ultrastructural level, of fine strctural cytochemistry, of chromatin spreading techniques, etc. And, last but not least, the recent tremendous advance of biochemical and molecular methods, including gene cloning and DNA sequencing, has also involved many protozoans as models.

However, the progress in protozoan karyology strongly depended also on modern ideas and new discoveries in general karyology and molecular genetics. Among such post-1950 theoretical advances one can list the discovery of the molecular structures of DNA and RNA, the identification of DNA as gene material, the discovery of the genetic code, the identification of RNA as information messenger, the understanding of the functions of most cell organelles and nuclear components. The discoveries of such phenomena as gene amplification and "processing" of the genome also have direct bearing on protozoan karyology. The distinction drawn between prokaryotic and eukaryotic cells is also a major theoretical achievement of the recent decades. Of course there are many other quite new discoveries, like that of mobile genes, which are important for protozoan karyology but too numerous to be listed here.

During the last 35 years, the vast majority of the protists (including the protozoa) proved to be typical eukaryotic cells. However, the dinoflagellates proved to differ from typical eukaryotes because they have many prokaryotic features in the structure of their chromosomes; they can therefore be called mesokaryotic organisms, as proposed by J.D.Dodge.

NON-DIVIDING NUCLEI

The fine structure of the non-dividing nuclei has been

studied during the last three decades in at least several hundred protozoan species (review: Raikov 1982). They proved to be surrounded by a classical nuclear envelope, which is, however, sometimes complicated by either differentiations of the nuclear lamina (e.g., the "honeycomb layer" in Amoeba proteus) or by extensions of the nuclear membranes themselves (e.g., in certain gregarines). The nuclei contain partly condensed, partly dispersed chromatin as well as typical nucleoli.

The technique of chromatin spreading has revealed that the chromatin of all protozoans except the dinoflagellates is organized into typical nucleosomes. Transcription complexes (especially nucleolar ones) have been visualized in Physarum or in the macronuclei of some ciliates (Tetrahymena, Paramecium, Bursaria). In Amoeba proteus, the non-nucleolar transcription complexes consist of very long helical RNP fibrils, the so-called nuclear helices.

STRUCTURE OF THE NUCLEAR GENOME

Nothing except the chromosome numbers in some species was known about the protozoan genome prior to 1950. Most data belonging to this field date from the seventies and eighties.

The absolute DNA content of the nuclei has been measured in many protists. It proved to vary within very wide limits, the haploid DNA content (c) being less than 0.1 pg in some trypanosomes and malaria parasites, but more than 100 pg in certain dinoflagellates. Here I am purposefully omitting the nuclei which are likely to be polyploid (Amoeba proteus), and the ciliate macronuclei where the genome is processed and amplified so that the nuclear DNA content may exceed 1000 pg.

The quantity of repetitive sequences in the protozoan genomes is usually within 5% to 60%. Typically eukaryotic is also the distribution of the repetitive sequences into several classes - highly, moderately, and weakly repetitive ones. The genes which are usually repeated in the eukaryotic genome are repetitive also in the protozoan ones: the ribosomal RNA genes, the genes for tubulins, actin, histones, and some others. As most eukaryotes, many protozoans (Euglena, the trypanosomids, Dictyostelium, Plasmodium, ciliate micronuclei)

display highly repetitive (satellite) DNA fractions with no known function. There are also short highly repetitive oligonucleotides localized in the chromosomal telomeres, e.g. in the kinetoplastids or in ciliate micronuclei (Blackburn 1984).

The genes for rRNA are usually integrated into the chromosomes and tandemly repeated. But in some cases they proved to be extrachromosomal and amplified, for instance, in the slime molds Physarum and Dictyostelium (Weiner and Emery 1982). This is also typical of ciliate macronuclei (reviewed by Raikov 1982).

Quite recently, transposable (mobile) genes have been discovered in the trypanosomids (review: Bernards 1985). The African trypanosomes (Trypanosoma brucei) are known to periodically replace their antigenic surface glycoproteins. It has been discovered that such antigenic switches are due to extra duplication of one or another pre-existing but silent gene coding for a certain kind of glycoprotein, and to transposition of its extra copy into a telomeric expression site.

DIVERSITY OF MITOSIS

Perhaps the most impressive advance of protistan karyology in the last 25 years is the comparative fine structural study of mitosis in a vast number of species (reviews: Heath 1980, Raikov 1982). The fine structure of mitosis has been investigated in almost all major protozoan taxa (with the curious exception of the myxosporidians). This allowed to understand for the first time the course of mitosis in coccidians, haemosporidians, trypanosomids, microsporidians, etc., and in many algae and fungi as well. Even in species with large mitotic figures, electron microscopy permitted to solve many ambiguities concerning the behaviour of the nuclear envelope, spindle fibres, kinetochores, and even chromosomes.

Pre-1950 schemes of classification of mitosis did exist, but of course they were based solely on light microscopy and could not take into account the mitotic patterns of small size. The first classification of protozoan mitoses based on fine structural data was published by A.Hollande in 1972; it was emended and supplemented by Raikov (1982), and the likely

ways of evolution of mitosis were outlined. A more complex classification of mitotic systems in the protists (especially fungi) was proposed by I.B.Heath (1980), who also advanced a hypothesis of evolution of mitosis. The two hypotheses have some points in common and some divergencies as well. For instance, it is not yet known what variant of closed intranuclear mitosis should be considered the most primitive - that with a straight static central spindle, like in many fungi (as suggested by Heath), or two independent half-spindles, like in the microsporidians (as suggested by Raikov).

Anyway, the mitotic patterns of the lower eukaryotes proved to be much more diverse than those of either metazoans or higher plants. Mitosis often proceeds here inside an intact nuclear envelope, and this seems to be a primitive condition. The functions of spindle microtubules apparently evolved from purely static ones (anchoring of the chromosomes to certain points of the nuclear envelope) to dynamic ones (transport of the chromosomes towards the poles and/or elongation of the mitotic spindle itself). And the great diversity of polar structures (spindle plaques, nucleus-associated organelles, etc.) shows that the centriole only secondarily became associated with the mitotic apparatus, an idea of J.D.Pickett-Heaps.

MESOKARYOTIC NUCLEI

During the period under review, the necessity has been recognized to separate the dinoflagellates from other protists as a unique mesokaryotic level of organization of life. This idea has originated from the early electron microscopic studies of the dinoflagellate chromosomes by P.Giesbrecht, H.Ris, E.Kellenberger, and others, who noticed that the unit fibrils (genofibrils) of these chromosomes were much thinner than in eukaryotes and resembled those of the prokaryote nucleoids. Also, J.D.Dodge has shown that these chromosomes contained no cytochemically detectable histone. All this has led Dodge to suggest that the dinoflagellates belonged to a special mesokaryotic level of organization, distinct from both prokaryotes and typical eukaryotes. In modern terms, the dinoflagellates have no chromatin: there are no nucleosomes on their

DNA filaments and their chromosomes contain at least 10 times less basic protein than the chromosomes of eukaryotes. Their basic protein is moreover not a real histone. All this shows that the mesokaryotes are apparently a group which deviated very early from the trunk of eukaryotes (Herzog et al. 1984).

In the last decades, the fine structure of the unusual dinoflagellate chromosomes which show a highly ordered periodic structure was studied in much detail. The early hypotheses of organization of these chromosomes favoured their polyteny (the models of P.-P.Grassé, of O.K.Haapala and M.-O. Soyer). But recent biochemical and genetic data have demonstrated that the dinoflagellate nucleus is basically haploid and that its chromosomes cannot be polytenic. Therefore, recent models of organization of these chromosomes (Oakley and Dodge 1979, Spector et al. 1981) assume their single-strandedness. But the exact structure of the dinoflagellate genetic apparatus is still an open question.

The mechanism of mitosis in dinoflagellates, which seemed very different in free-living and parasitic forms in the era of light microscopy, proved to belong to one and the same type which can be called dinomitosis, using Chatton's term. Electron microscopic studies, especially by D.Kubai, H.Ris, B.Oakley, and J.D.Dodge, have shown that the spindle is here always extranuclear and the nuclear envelope intact, so that microtubules interact with the chromosomes via the kinetochores which are literally built into the nuclear envelope. The spindle is often cryptic, hidden inside cytoplasmic channels piercing the nucleus; that is why it could not be detected with the light microscope. The difference between free-living and parasitic forms is only that the former usually have many channels while the latter have only one (review: Raikov 1982).

NUCLEAR DUALISM

The nuclear dualism has long been known to exist in the ciliates, but in the fifties it has been discovered by K.G. Grell in some foraminiferans as well (review: Grell, 1979). In the diploid agamonts of some Rotaliidae, several nuclei remain generative while one (rarely few) becomes somatic. The

latter is transcriptionally active but loses the ability to undergo meiosis, since its chromosomes cannot pair and attach to spindle fibres. In the rotaliids, the somatic nucleus remains diploid, but in some other foraminiferans (Cibicides) it can accumulate more DNA due to either polyploidy or gene amplification, as shown by M.N.Voronova. The differentiation of the somatic nucleus is irreversible, and generative nuclei are able to become somatic at any time. One can suspect that the genome of the somatic nucleus becomes modified during its differentiation as a result of a kind of genome processing, which would make the differentiation irreversible.

As to the ciliates, it has been shown by Raikov and colleagues since 1955 that at least 100 species of marine psammophilic ciliates belonging to the genera Trachelocerca, Trachelioraphis, Trachelonema, Remanella, Kentrophoros, and Geleia, as well as the few fresh-water species of Loxodes, have a nuclear apparatus of a quite unusual kind. These ciliates are all multinucleate (with at least two somatic nuclei), and their somatic nuclei (or macronuclei) proved to be about-diploid in DNA content. Moreover, such macronuclei are unable to divide at all. Therefore, they must be regularly produced anew from generative nuclei (or micronuclei) in every cell cycle, in order to supplement the number of existing macronuclei. Here again, a processing of the macronuclear genome is likely to occur, making this nucleus active in transcription but unable to divide due to the loss of some elements of the genome (see Raikov 1982, 1985).

Much electron microscopic work has been done with the nuclei of these ciliates, which are now considered to constitute an order or even class of their own, the Karyorelictida. The fine structure of their macronuclei proved to be typical of actively transcribing nuclei, while that of the micronuclei was typical of inert nuclei (Raikov 1985).

The appearance of the nuclear dualism in evolution apparently opened wide possibilities for the rearrangement of the genetic apparatus of the somatic nucleus, to make it a most efficient transcription-directed apparatus. This is possible because the other (generative) nucleus always retains the full

genome and thus remains able to assure the genetic continuity as well as the regular renewal of the somatic nuclei. This peculiarity of the nuclear dualism is even more conspicuous in other ciliates which have "polyploid" macronuclei.

"POLYPLOID" MACRONUCLEI

The theory of polyploidy of the typical (DNA-rich) ciliate macronucleus has just taken shape by the year 1950, due to the works of G.Piekarski, L.Geitler, T.M.Sonneborn, and K.G.Grell. It was postulated that the macronucleus contained the same genome as the micronucleus but in hundreds of copies, and that perhaps the chromosomes of each genome sticked together into a kind of subnuclei or composite chromosomes. This theory was widely accepted until the late sixties.

During the last 15 years however, the polyploid theory has been thoroughly revised. It has become clear that there is no universal type of organization of the macronucleus common to all ciliates. This followed from the new morphological and especially biochemical and molecular research.

D.Ammermann was the first to demonstrate that the chromosomes of the developing macronucleus of Stylonychia initially become polytenic, and then fragment into short sections or chromomeres. Later, it has been shown in both D.Ammermann's and D.M.Prescott's laboratories that the macronuclei of all hypotrichs have completely lost chromosomal organization; instead, their DNA is in the form of gene-sized molecules which possess specific telomeric sequences and are capable of both replication and transcription. They can thus be termed minichromosomes (reviews: Raikov 1982, Prescott 1984). It has also been shown that there is a massive DNA diminution during macronuclear development in the hypotrichs, which results in a 10 to 100-fold reduction of the size of the macronuclear unit genome as compared with the micronuclear haploid genome. But the remaining reduced genome is repeated in the macronucleus thousands of times. Such macronuclei evidently cannot be called polyploid any more; the term "ampliploid" has been coined for them by V.Schwartz.

In other ciliates however, the processing of the macronuc-

lear genome, though existing, is not so dramatic. For instance in Tetrahymena about 10% of the micronuclear DNA sequences are lost during macronuclear development, and this corresponds to about 5000 sites of deletion in the entire genome (Yao et al. 1984). But the fragments of the chromosomes thus produced then re-unite into chromosomes, except the ribosomal RNA gene sequence which is excised and remains extrachromosomal. The macronuclei of this type consequently contain chromosomes (though modified ones) which become replicated many times. Such a nucleus can still be called polyploid. Perhaps, most ciliates (Paramecium, Stentor, etc.) belong to that type.

Finally, a third group seems to exist, where the macronucleus also contains chromosomes which, however, aggregate into clusters corresponding to diploid subnuclei. This type of macronuclear structure, which can be called polygenomic or subnuclear, occurs in the Colpodida and the Nassulida.

Thus, the evolution of the somatic nuclei has progressed much further in the "typical" ciliates than in the karyorelictids. Not only each gene has been extensively multiplied, but also the processing of the initial genome is here much more pronounced, especially in the hypotrichs. It is obvious that such rearrangements of the genetic apparatus are impossible without nuclear dualism since they would seriously impair the stability of the macronuclear genome. But due to the micronucleus the ciliates always conserve a full normal genome capable of replacing the worn-out macronucleus with a new one.

In conclusion, one can state that the recent 35 years have led to impressive advances in the understanding of both the structure and functions of protozoan nuclei. But the foundations of this progress were laid down still in J.Gelei's time. In the recent period, the karyology of the protists remained largely morphological. This period can even be described as the era of electron microscopy. But the situation is rapidly changing just now, and one may presume that the next era will be that of molecular genetics and biochemistry of protozoan or better protistan nuclei.

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INTERSTRAIN DNA POLYMORPHISM DETECTED IN *WILLAERTIA MAGNA* (VAHLKAMPFIIDAE) BY RESTRICTION ENDONUCLEASE DIGESTION

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SUMMARY

The genomic DNA of 8 *Willaertia magna* strains from 4 different continents was digested with 6 different restriction endonucleases and the fragments were separated in horizontal agarose electrophoresis. With Pst I digestion a homogeneous DNA fragment pattern was obtained in the 8 *Willaertia* strains which was totally different from *Naegleria*. The patterns obtained with Eco RI, Alu I and Bam HI digestion were typical for each strain although some common bands were observed in each strain.

INTRODUCTION

Identification of free-living amoebae by morphology is only possible at the genus level. With serological methods species can be defined but even then many cross-reactions are observed. Rapid and reliable identification of species, subspecies and even strains has been possible by the use of isoenzyme analysis (De Jonckheere 1982, Nerad and Daggett, 1979). Recent advances in DNA technology now provide a new tool for the identification of organisms. Restriction enzymes have been used on mitochondrial DNA for the classification of *Acanthamoeba* (Bogler et al. 1983, Costas et al. 1983). This paper describes the first results obtained with restriction endonuclease treatment of the genomic DNA of free-living amoebae. The genus *Willaertia* was chosen as the first target for this technique as there is only one species known while a set of 8 strains from 4 different continents is available for comparison. The genus *Willaertia* is important as the organism has the same maximum growth temperature and is encountered in the same environment as *Naegleria fowleri*, which causes

primary amoebic meningoencephalitis in man. This investigation forms a basis for applying the DNA technique on Naegleria spp.

MATERIALS AND METHODS

Strains

The two reference strains used in the original publication on the genus definition of Willaertia (De Jonckheere et al. 1984), Z503 and T5(S)44, are from Europe. Strains 002 and 009 were isolated in Australia, strains NJ4 and NJ13 in India, while C2C45A and C2C45D came from Mexico.
The isolates were identified by immunofluorescence and/or isoenzyme analysis as belonging to W.magna. Minor differences were observed in the isoenzyme patterns of different strains.

Cell growth and DNA isolation

Each strain was grown in 10 Roux bottles each containing 50 ml of medium : SCGYEM (Z503 and T5(S)44) or YPNF (all others). After 48 h incubation at 30°C, the amoebae were dislodged from the wall by shaking and sedimented by centrifugation. The pellet of amoebae was suspended in 5 ml cold SE buffer (100 mM EDTA, 100 mM NaCl, 10 mM Tris, pH 7.8). Lysis was performed with 1 % sarkosyl and proteinase K (50 µg/ml) was added immediately. The suspension was incubated at 37°C for 3 h. DNA was extracted with a mixture of phenol : chloroform : isoamylalcohol (50 : 49 : 1) and precipitated with ethanol. DNA was dissolved in 2 ml 10 mM Tris, 0.1 mM EDTA and treated with 20 µg/ml RNase for 30 min at 37°C. Proteinase K (50 µg/ml) and 0.1 % SDS was added and the solution incubated for 30 min at 37°C. After extraction with phenol : chloroform : isoamylalcohol and ethanol precipitation the DNA was finally dissolved in 0.5 ml 10 mM Tris (pH 7.5).

Restriction enzyme analysis

Samples of 22 µl genomic DNA were digested with 2.5 U Pst I (Pharmacia-P-L Biochemicals), Eco RI, Sal I, Taq I, Alu I and Bam HI (Boehringer, Mannheim) in the appropriate low salt, medium salt or high salt mixtures (Maniatis et al. 1982) giving a final volume of 25 µl. After 1 h at 37°C restriction endonuclease-digested DNA samples were electrophorezed in 0.7 % agarose gels containing 0.5 µg/ml ethidium bromide. The gels were run horizontally at 90V at 4 h in Tris-borate buffer. Lambda DNA-Hind III/ΦX 174 RF DNA-Hinc II digest (Pharmacia P-L Biochemicals) was used as size marker.

RESULTS

Total DNA extracts migrated as 2 observable bands in agarose gel electrophoresis. The lesser band with lower molecular weight is probably mitochondrial DNA. When a procedure was used for isolation of plasmid DNA, denaturing linear DNA but not affecting covalently closed circular DNA (Takahashi and Nagano, 1984), only the band with lower molecular weight was recovered.

With Pst I digestion of total DNA the 8 Willaertia strains showed an identical pattern of 5 bands between 1,000 and 5,000 base pairs (Fig. 1,a). Some variation at higher base pair numbers can be seen only when electrophoresis was continued for a longer time. Genomic digestion with Alu I (Fig. 1,b) gave many bands that are similar for all Willaertia strains, with some variation especially at high base pair numbers (between 1,000 and 5,000). Digestion with Sal I and Taq I gave only very faint bands and are thus not useful for typing.

With Bam HI the 8 Willaertia strains all gave 2 typical bands at 20,000 and 9,000 base pairs respectively but the fragments with lower number of base pairs showed variation (Fig. 1,c). With Eco RI the 8 Willaertia strains showed two identical bands, one at about 4,000 base pairs and one at about 600 base pairs. A lot of variation was seen with other bands, the total number ranging from 7 to 12 bands (Fig. 1,d).

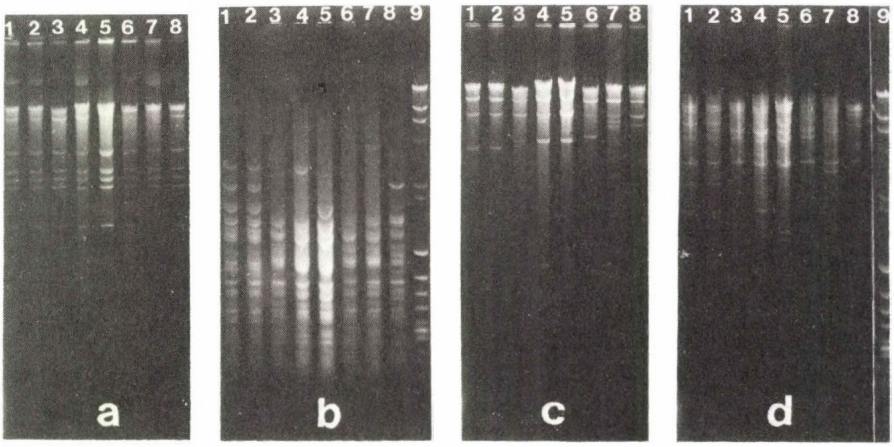

Fig. 1 Pst 1(a), Alu I(b), Bam HI(c) and Eco RI(d) digestion of genomic DNA separated in 0.7 % agarose gels (90V for 4 h). 1:C2C45D ; 2:C2C45A ; 3:NJ13 ; 4:NJ4 ; 5:T5(S)44 ; 6:Z503 ; 7:009 ; 8:002 ; 9:size markers.

DISCUSSION

The two Mexican isolates showed identical DNA patterns with all restriction enzymes investigated. This is not surprising as they were isolated from the same sample. With Bam HI digestion strain NJ13 from India is identical to strain T5(S)44 from Belgium, while strain NJ4 from India has one additional band with about 1,000 base pairs. With Eco RI and Alu I there are more differences between these three strains. Differences are observed with Bam HI, Alu I and Eco RI between each of the two European strains and the two Australian strains. They were isolated from different habitats.

A typical DNA pattern is obtained with Pst I digestion of all strains, which can therefore be used for the identification of W.magna. No difference in patterns was obtained when RNA was not eliminated.

With the restriction enzymes investigated, Naegleria strains gave patterns that have nothing in common with the Willaertia isolates. Therefore, restriction endonuclease patterns of genomic DNA seems to be very promising for the classification and identification of free-living amoebae. This is important for screening the environment as several species of Naegleria and Acanthamoeba cause severe disease difficult to cure and most often leading to death in man.

ACKNOWLEDGMENTS

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RELEASE OF LYSOSOMAL ENZYMES – ITS BIOLOGICAL SIGNIFICANCE IN A PROTOZOAN CELL

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INTRODUCTION

A variety of lower eukaryotes, mainly unicellular organisms, are known to release acid hydrolases into the surrounding culture medium: the slime mold Physarum polycephalum (Kuehn et al., 1977), the yeast Saccharomyces cerevisiae (Field & Shekman, 1980), the protozoa Leishmania donovani (Gottlieb, 1980), Dictyostelium discoideum (Pannell et al., 1982), Acanthamoeba castellanii (Hohman & Bowers, 1984), Colpidium campylum (unpublished own results) and Tetrahymena thermophila (Müller, 1967; 1972). Müller showed that the released acid hydrolases of Tetrahymena have lysosomal origin and that the enzymes are released constitutively, both into nutrient and non-nutrient medium. The biological meaning of release of lysosomal enzymes, however, has remained an enigma. That the released hydrolytic enzymes might function in extracellular predigestion of nutrients has been considered unlikely (Müller, 1972; Nilsson, 1979) because of the high dilution of the enzymes in the natural freshwater habitat of this ciliate.

I shall combine in the following literature data and own recent results suggesting that extracellular predigestion of nutrients could be a vital requirement for a parasitic life of Tetrahymena.

RESULTS AND DISCUSSION

Utilization and uptake of nutrients - the test tube situation

Tetrahymena is known as a suspension feeder ingesting bacteria and detritus particles in nature. In the laboratory, however, the ciliate is cultivated axenically on dissolved protein lysates or even on chemically defined medium. The analysis of mutants, unable to form food vacuoles has shown that Tetryhymena has two almost independent systems for nutrient uptake: (1) the food vacuoles for uptake of particulate matter and (2) the plasma membrane for uptake of dissolved low-molecular-weight material (Rasmussen & Orias, 1975). The action of the released lysosomal enzymes may provide the cell with these small components by hydrolyzing poly-(oligo-)meric organic compounds into the monomeric ones suited for plasma membrane uptake. Rasmussen et al. (in press) have shown that phosphate is indeed liberated from organic phosphate esters (like α-glycerophosphate) outside the cell. Since these compounds are neither spontaneously hydrolyzed in the medium, nor do permeate through the cell membrane these compounds have to be split by released phosphatases. Rasmussen et al. have shown that the food-vacuole-less mutant maintained in defined medium without free orthophosphate utilizes phosphate esters as a phosphate source even in the absence of food vacuole formation. Tetrahymena, therefore, exploits extracellular digestion of nutrients for its multiplication, because of its ability to secrete the appropriate acid hydrolases.

Release of lysosomal enzymes - a secretory process

The notation of a constitutive release of acid hydrolases even into inorganic starvation medium has been used as an argument to question a function of the released enzymes in predigestion of nutrients (Nilsson, 1979). In agreement with this view the cytoproct has been proposed as the cellular site, where the hydrolases become released together with debris from digestive vacuoles (Blum & Rothstein, 1975). This proposal is based on the correlation of an increased release of two acid hydrolases together with a pharmacologically induced enhanced rate of food vacuole egestion (Rothstein & Blum, 1974). We have

recently shown that the release of lysosomal enzymes is a secretory process: release of lysosomal enzymes (RLE) can be increased specifically by Ca^{2+} mobilizing drugs (Tiedtke, et al., 1984). The specificity of RLE was proven by the non-release of a sensitive cytosolic marker enzyme, isocitrate dehydrogenase (Fig. 1).

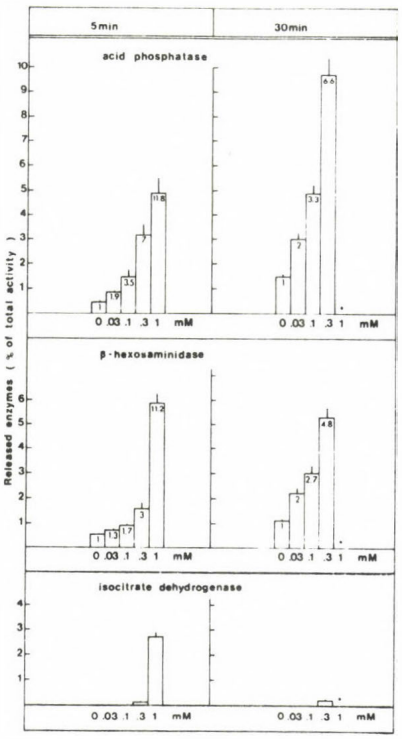

Fig. 1. Enzyme release in response to incubation with dibucaine. Cell suspensions were incubated with the drug for 5 min and 30 min in 10 mM Tris-Cl, pH 6.0 Enzyme activities of cell-free supernatants are expressed as percentages of the total cellular enzyme activities. The numbers inside the columns indicate the increase in enzyme release with respect to the control (=1). *Cell lysis: data not plotted.

The experimental proof of RLE as a specific secretory process is a crucial precondition for considerations on a biological significance of RLE in the life of this ciliate.

Our experiments have also shown that defecation and secretion of lysosomal enzymes are unrelated processes, since RLE can be increased considerably in spite of an unchanged rate of food vacuole egestion. These observations have been confirmed recently by the use of a mutant of Tetrahymena unable to secrete lysosomal enzymes (sec^--mutant). This mutant egests food vacuoles in the same rate as the wildtype but releases less than 5% of the lysosomal enzymes activities secreted by the wildtype (Tiedtke, et al., unpublished results).

Preadaptions to parasitism

Parasites and scavengers of the genus Tetrahymena

The claimed ineffectivity of released lysosomal enzymes in predigestion of nutrients is based on the notation that Tetrahymena lives, usually in small numbers in freshwater ponds or lakes. A closer examination of the literature reveals that parasitism or facultative parasitism is observed in many species of Tetrahymena (Table 1).

Table 1. Parasitic forms of Tetrahymena

Parasite	Host	Reference
T. limacis	Monadenia fidelis Prophysaon andersoni (Gastropoda,Pulmonata)	Kozloff,E.N.,1956
T. chironomi sp.nov.	Chironomus plumosus	Corliss,J.O.,1973
T. pyriformis T. corlissi	Polycelis felina Crenobia alpina (Platyhelminthes, Turbellaria)	Wright,J.F.,1981 "
T. rotunda n.sp.	Simulium sp.	Lynn,D.J. et al. 1981
T. dimorpha n.sp.	Simulium equinum	Batson,B.S.,1983

The most frequent hosts are aquatic larvae of insects and their imagines but also other aquatic invertebrate and vertebrate hosts have been reported (Corliss, 1973). Other forms of the genus Tetrahymena have been observed as scavengers (Corliss, 1973). Both as parasites and scavengers Tetrahymena lives and grows in microenvironments rich in organic compounds similar to the test tube condition. The hydrolytic cleavage of organic compounds into readily usable monomers is in our view, a function of release of acid hydrolases.
Eisen and Tallan (1975) have shown that Tetrahymena releases a papain-like protease, which cleaves antibodies directed against the ciliate into univalent fragments. The ciliates thus are able to free themselves from the immobilizing action of antisera. The univalent antibody fragments remain bound to the cell surface, mask the ciliates and prevent their detection by the host's defence system.
Acid hydrolases released into microenvironments and/or associated with the cell surface therefore may have vital functions in parasitic or scavenging Tetrahymena.

Adaptations for invasion of hosts

Although we do not know how parasitic Tetrahymena enter the insect host's hemocoel - e.g. per os or through the cuticle of molting larvae - the ciliates are equipped with enzymes capable of cleaving chitinous bonds: secreted N-acetyl-ß-D-hexosaminidase has been purified and shown to cleave the chitin-type bonds (Tiedtke, 1983a). The cooperative action of this and other hydrolases thus may facilitate the penetration of the parasite into the hosts body. Recently, Florin-Christensen et al. (1985) have identified cytolytic proteins released into the medium, as phospholipase C. Acid phospholipases lyse erythrocytes and may liberate organelles and soluble nutrients from host tissues. Tetrahymena itself posesses phosphono-lipids in its membrane that protect hydrolysis of the own membrane by released phospholipases. Two hydrolases have been shown to be associated with the cell membrane, peptidase (Zdanowski & Rasmussen, 1979) and N-acetyl-

ß-D-hexosaminidase (Tiedtke, 1983b). The latter enzyme covers the entire cell surface as revealed by specific immunostaining (Fig. 2).

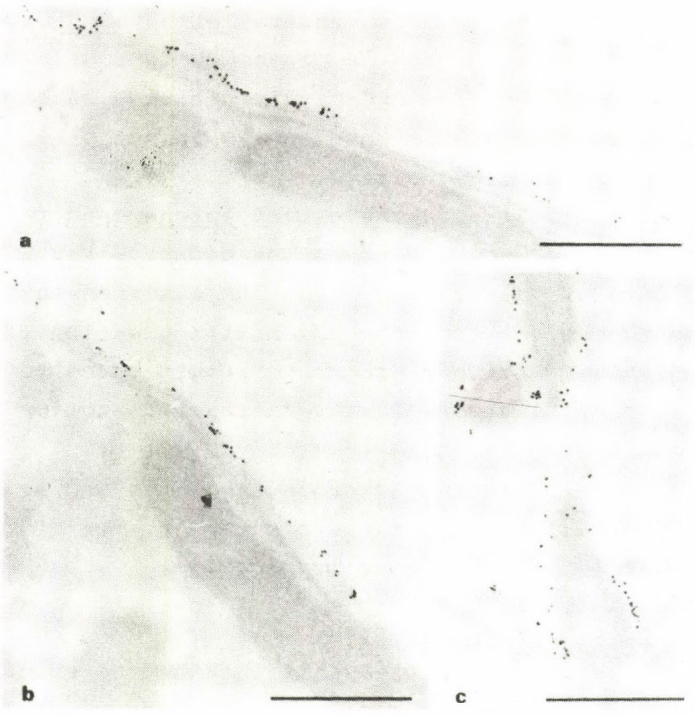

Fig. 2. N-acetyl-ß-D-hexosaminidase on the cell surface of Tetrahymena thermophila. Indirect immunostaining, with monospecific serum against ß-hexosaminidase as first and colloidal gold-anti-rabbit-IgG as a second antibody. Bar: 0.5 um.

CONCLUSIONS AND SUMMARY

The presented arguments let us propose that release of lysosomal enzymes is a meaningful phenomenon in the biology of Tetrahymena:

1) The release of lysosomal enzymes is a specific secretory event: it depends on Ca^{2+} ion mobilization and takes place selectively.

2) Tetrahymena has two almost complete nutrient uptake systems: (a) the cytostome-food vacuole system for uptake of particulate material and (b) the cell membrane for uptake of low-molecular-weight components.
3) The ciliate hydrolyzes outside the cell phosphate from organic phosphate esters, which are neither spontaneously hydrolyzed in the medium nor permeable to the cell membrane. Tetrahymena can grow on media supplemented with organic phosphate esters in the absence of food-vacuole formation.
4) Many members of the genus Tetrahymena are parasites or scavengers. The parasitic life offers microenvironments, where effective enzyme concentrations can accumulate.
5) Tetrahymena possesses the enzymatic outfit for invasion into and attack of insect hosts: ß-hexosaminidase, that splits chitin-like bonds and phospholipases, that lyse cell membranes. The former enzyme and a peptidase are also found associated with the cell surface.
6) A papain-like protease is cell surface associated and released into the medium. It splits antibodies into univalent fragments and helps Tetrahymena to escape the host's defence system.

Food-vacuole-less mutants and those blocked in secretion of lysosomal enzymes are presently used to show that Tetrahymena exploits and most likely depends on secreted lysosomal enzymes for its nutrition.

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SELECTION AND CHARACTERIZATION OF TETRAHYMENA MUTANTS BLOCKED IN SECRETION OF LYSOSOMAL ENZYMES

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INTRODUCTION

The ciliate Tetrahymena thermophila releases large amounts of lysosomal hydrolases constitutively into the extracellular medium (Müller, 1967; 1972). In nutrient medium there is a permanent accumulation of released enzyme activities in the medium while the cellular content of enzyme activities remains unchanged. Cells suspended in starvation medium release within 5h as much as 80% of their initial present cellular enzyme activities of some glycosidases into this medium, leaving the cells with 20% of their cellular enzyme activities.
Due to the constitutive nature of lysosomal enzyme release (RLE) and the high dilution of the enzymes in the natural environment any biological significance of RLE has been questioned (Müller, 1972; Nilsson, 1979). Another unsolved question is the transport route of the released enzymes through the cell and across the cell surface to the extracellular medium.

With the help of mutant's blocked in release of lysosomal enzymes we hope to answer some of these questions. In this report the isolation of secretion deficient mutants (sec^-) and the genetic and phenotypic characterization of one of these mutant lines are presented.

MATERIAL AND METHODS

Strains: Cu 399, a functional heterokaryon (Bruns & Brussard, 1974) of strain B and the C*-strain of Tetrahymena thermophila were used in this study.

The sec⁻-mutant MS-I was derived from Cu 399 after chemical mutagenesis followed by short circuit genomic exclusion (Bruns et al., 1976).

Culture: Cells were grown in PPYS-medium (1% Proteose Peptone, 0.1% Yeast Extract, 0.003% Sequestren) at 30°C to cell concentrations of 3-5x10^5 cells/ml.

For the secretion experiment cells were sedimented, washed once with Dryl's solution and then incubated in fresh Dryl's solution at 30°C. Cell free supernatant was collected after sedimenting the cells at 2.500 rpm for 3 min (Hettich Roto-fix II). The cell sediment and remaining medium was homogenized by freeze-thawing and sonication for 30 sec at 15 mamp. The 10.000 x g supernatant was used for estimation of the cellular enzyme activities.

Mutagenesis and screening: Cu 399 was mutagenized with NG (10 mg/ml of N-methyl-N'-nitro-N'-nitrosoguanidine) for 2-4 hours (Orias et Bruns, 1976) and then mated with C* to induce short circuit genomic exclusion.

The supernatants from cycloheximide resistant clones (i.e. whole genome homozygous clones) were screened with fluorogenic substrates for detection of clones with deficiencies in secretion of β-hexosaminidase (EC 3.2.1.52) and acid phosphatase (EC 3.1.3.2) at both 30°C and 37°C.

Enzyme assays: Enzyme activities were assayed at 37°C using the appropriate p-nitrophenyl-substrates for the glycosidases and acid phosphatases as described elsewhere (Tiedtke, 1983) DNAse was measured according to De Duve et al., 1955. The protein content was determined by the method of Lowry.

RESULTS

Two clones derived from short circuit genomic exclusion exhibited stable deficiencies in release of lysosomal enzymes when

grown at 30°C and 37°C. In the following one of these lines, MS-I, is described in detail.

Deficiencies in release of lysosomal enzymes

A comparison of the secretion characteristics of wildtype (Cu 399) and the sec⁻-mutant (MS-I) is shown in Fig. 1. Indicated are the enzyme activities in the supernatant compared to the activities present in the total system (cells plus medium) after four hours of incubation in starvation medium.

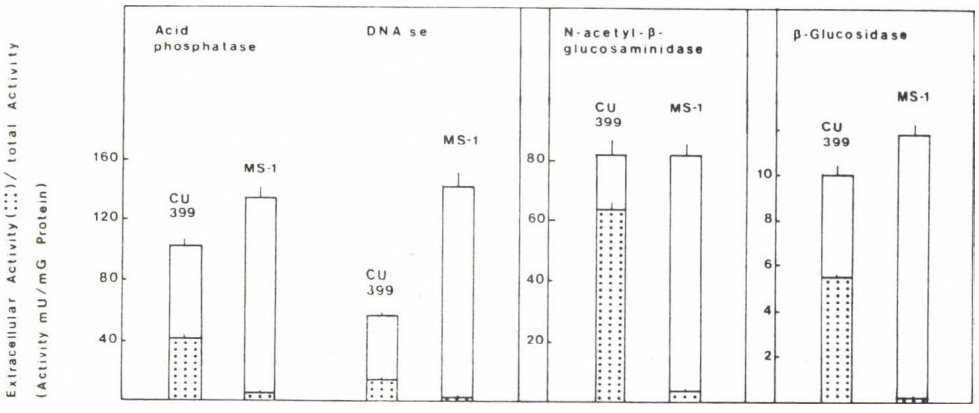

Fig. 1: Secretion of lysosomal enzymes in wildtype (Cu 399) and a sec⁻-mutant (MS-1) of Tetrahymena thermophila, blocked in secretion of lysosomal enzymes, during 4h of starvation in Dryl's solution. The ratio of secreted enzyme activities versus the corresponding total activities (cells plus medium) is indicated. Mean and standard deviation of N=4 experiments are shown.

Cu 399 shows the typical secretion rate of the wildtype, i.e. releasing about 50-80% of the glycosidases activities and about 20-40% of acid phosphatase and DNAse activities to the medium. The sec⁻-mutant MS-I is almost totally blocked in release of these lysosomal hydrolases and also all other hydrolases (data not shown) as far as it has been investigated. A background release of less than 5% of the total cellular β-hexosaminidase, β-glucosidase, DNAse and acid phosphatase activities, however, is observed.

The secretion kinetics for β-hexosaminidase and acid phosphatase of the wildtype (Cu 399) and the sec⁻-mutant, MS-I are shown in Fig. 2. Even on a longer time scale in MS-I there is just a base line of enzyme release.

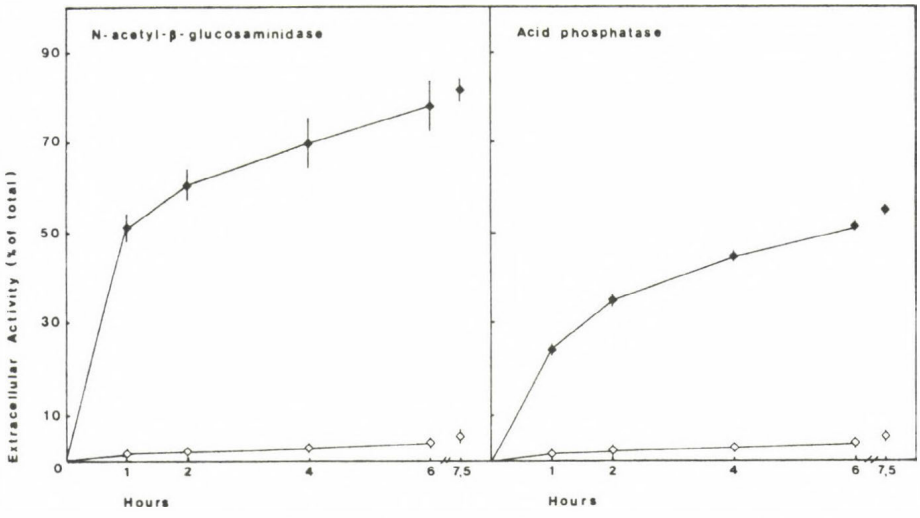

Fig. 2: Secretion kinetics of wildtype (Cu 399), black symbols, and the sec⁻-mutant (MS-1), open symbols, of Tetrahymena thermophila. Mean and standard deviation were calculated from 4 experiments.

Genetical analysis of the sec⁻-mutant,MS-1

The genetical analysis of MS-1 shows that a single gene mutation is responsible for the deficiency in hydrolase secretion in MS-1 (Data not shown). The mutation is recessive; backcrosses were performed and non secreting F_2 lines were obtained in ratios expected for a mendelian distribution of a single recessive allel. The genetical analysis showed that an altered morphological feature, of MS-1, expressed in dilute salt media, is directly correlated to the secretion deficiencies.

Morphological characteristics of the sec⁻-mutant:

Whereas in nutrient medium MS-1 has wildtype morphology, upon starvation in Dryl's and other starvation media it exhibits a rather dramatical change in cell shape.

While the wildtype (Cu 399) shows the normal features of a starved cell, a slim cell body with only a few food vacuoles (Fig. 3a) the sec$^-$-mutant develops after 4 hours of starvation some (3-10) voluminous vacuoles which progressively enlarge and finally after 5-12 hours fuse to one big vacuole (Fig. 3b).
In this final stage the cell itself resembles a big vacuole surrounded by a rim of cytoplasm and organelles.

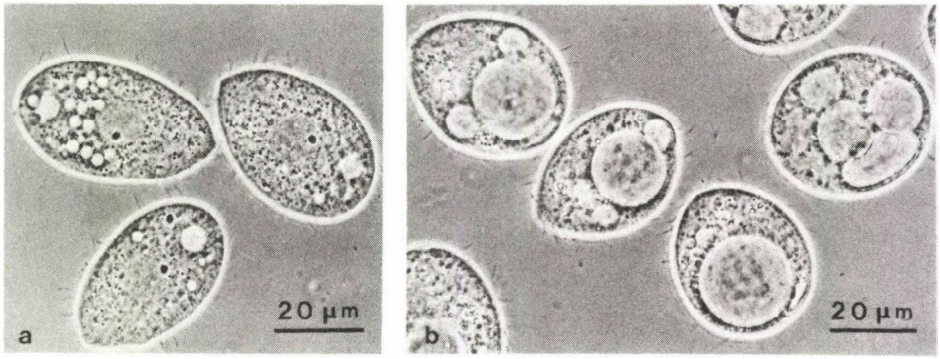

Fig. 3: Micrographs of living, slightly compressed Tetrahymena thermophila (electron flash). The cells were incubated in Dryl's solution for 6h. Wildtype (a) and a sec$^-$-mutant(b).

Transferred to nutrient medium (or to a medium of higher osmolarity), the cells retain their normal cell shape after 4 hours. These observations suggest that the sec$^-$-phenotype is correlated with an apparent deficiency in osmoregulation. It should be noted however, that size and pulsation frequency of the contractile vacuole seem not to be affected either in nutrient or in Dryl's medium.

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Symposia Biologica Hungarica 33 (1986)

HOW AND WHY DO RECEPTORS DEVELOP IN THE TETRAHYMENA IN PRESENCE OF HORMONES?

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About 12 years ago we demonstrated for the first time that the unicellular Tetrahymena was able to respond to, or to bind, hormones of higher organisms (1). Naturally, the responses observed often differed from those shown by the adequate target cells, which are part of specialized organs. However, in certain cases the Tetrahymena responded exactly as the target cells, to insulin(2) and adrenalin(3) by altering its glucose metabolism, to histamine and serotonin (1,4) by changing its phagocytic activity, and to thyroxine and its precursors by growth increase. In the case of other hormones the same indexes were part of the general response, which may also become expressed by other functional changes, such as increase or decrease in RNA synthesis, etc. (6,7). The response of the Tetrahymena to a hormone is usually less pronounced at the primary interaction than at reexposure(s), but it is nevertheless fairly selective. For example, the Tetrahymena can differentiate serotonin from the chemically related plant hormone indole-acetic acid and its derivatives, e.g. methoxy-tryptamine(4), and shows quantitative differences in response to certain members of the thyroxine and serotonin series, being most responsive to diiodotyrosine (T_2) and to serotonin itself, respectively.

The primary interaction with the hormone alters the responsiveness of the Tetrahymena radically, as judged from the considerable increase in hormone binding capacity or intensity of response to reexposure(s) (8-12). A single interaction accounts for persistence of the altered binding capacity and res-

ponse (Fig.1) over as many as 500 offspring generations(13). Primary interaction thus evokes a fundamental biological response, which has been termed by us a "hormonal imprinting",for the event of primary interaction becomes imprinted, and the "memory" of it is transmitted, to many subsequent generations.

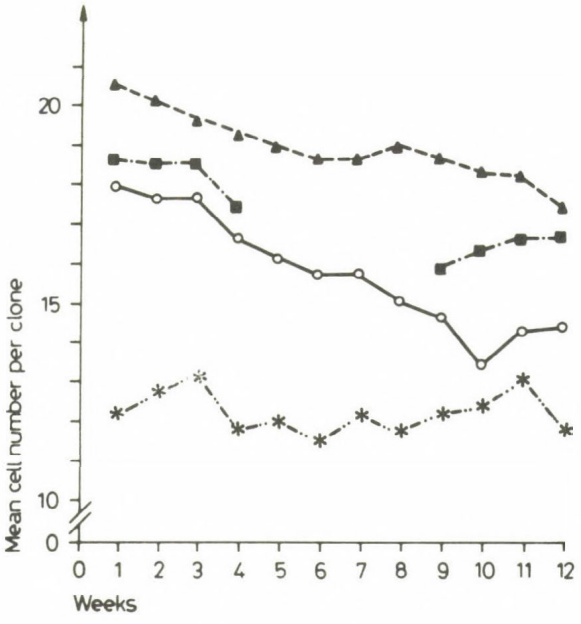

Fig.1.Mean rate of multiplication of Tetrahymena populations under different experimental conditions ▲ =subcultures treated repeatedly once a week with T_2; ■ =subcultures from the same stock as used for repeated treatment, but treated only once with T_2,at the beginning of the experiment;o=subcultures from another stock, treated only once with T_2, at the beginning of the experiment; * =control stock(not treated).

Evidence of presence of several hormones characteristic of higher organisms in the Tetrahymena (14) cannot in itself solve the problem why the unicellular should respond to these hormones. Another question, which remains to be answered, with special regard to the supposed involvement of imprinting in hormone and receptor evolution, is how the information received by primary interaction with the hormone becomes fixed for a memory-like transmission.

Investigations into the binding specificity of mammalian receptors are based on experiments with 10^{-8}-10^{-9}M concentrations of hormone. The Tetrahymena, too, responds well to these concentrations, which are also suitable to induce imprinting. The time required for the establishment of imprinting is 1 to 4 h; no durable imprinting occurs, if hormone exposure lasts less than 1 h (15). Lower concentrations, ranging between 10^{-12} and 10^{-15}M, can still evoke a - relatively weaker - response, but 10^{-18}M cannot. The latter concentration is nevertheless sufficient to induce imprinting. Tetrahymenas exposed to 10^{-18}M T_2 proved to be irresponsive, yet acquired a "memory" of the primary interaction, to judge from the measurable increase in the response of their offspring generations. It follows that the Tetrahymena is highly sensitive to hormone-like molecules, and can therefore "remember" interaction with very low concentrations, if interaction with these lasted long enough. In this light the time factor seems to play a greater role in hormonal imprinting than the concentration of the hormone; this interrelationship can throw a light on several problems to be discussed later in this review.

Experiments based on exposure of Tetrahymena to 10^{-18}M hormone (which is low enough to present practically only a single hormone molecule for each cell) made possible the comparison of the "memory" of the Tetrahymena to neuronal memory. Exposure to 10^{-18}M hormone (Table 1) for 4 h did induce imprinting,

Table 1. Mean growth rates of Tetrahymena cultures reexposed to T_2 1-week after 1x4 and 4x1^{h} preexposure to T_2

		Percentual difference
1 × 4	17.85	43.3
Control	12.45	
4 × 1 h	20.95	76.0
Control	11.9	

but to a lesser degree than four successive 1-h exposures with intervals in between (16). Thus - by analogy of the neuronal memory, - repetition enhanced the intensity of imprinting. Similar results were obtained in studies of retroactive interference, i.e. on exposing the cells simultaneously to several other hormones immediately after imprinting with a given hormone (e.g. T_2). The influence of the other hormones abolished T_2-induced imprinting to a considerable degree, although they did not themselves induce a "memory" (Table 2). This indicates that the "memory" of the Tetrahymena, referred to as imprinting, bears a close resemblance to neuronal memory, although they certainly are not identical in every respect.

Table 2. Mean growth rates of Tetrahymena cultures preexposed to hormones, hormone-like material, or a combination of these after 4h preexposure to T_2

1st treatment (preexposure)	none		T_2															
2nd treatment (1st reexposure)	-		T_2	T_2	serotonin		gramine		epinephrine		histamine		dopamine		combination			
	12.5		14.8	19.4	19.5		12.5		12.2		14.4		14.5		14.4			
3rd treatment (2nd reexposure)	-		T_2	T_2	T_2	sero-tonin	T_2	gramine	T_2	epi-nephrine	T_2	hista-mine	T_2	dopa-mine	T_2	combi-nation		
	12.1		18.2	20.0	18.2	19.1	15.8	14.0	13.9	14.0	16.5	16.3	15.0	14.0	12.2	11.6		

The mechanism of hormonal imprinting is still not fully understood. The intact functional state of the cell membrane is in all probability an essential prerequisite of normal imprinting. Any interference with the normal functions of the cell membrane, as e.g. rendering it rigid by incorporation of lipid-like (ergosterol, etc.) molecules (17), or more fluid by thermic effects (cooling or heating) or chemical effects (phenothiazines, local anaesthetics) (18), hampers the normal course of imprinting. Inhibitors of membrane protein movement (clustering) have a similar effect (19). It is self-evident that the functioning, or even the formation of receptors, presupposes an intact functional state of the cell membrane. Inhibition of cellular protein or DNA synthesis also hampers the normal course of imprinting (19), and so does any disturbance of cellular Ca-

metabolism. Ca-mediation seems to play a primary role in imprinting, presumably by involvement of the Ca-calmodulin system (20) in the latter's mechanism. The mediator role of cAMP seems to be less important that of Ca-calmodulin.

The foregoing considerations indicate that the imprinting mechanism involves many functions of the cell, and can therefore be adversely affected - although to different degrees - by the impairment of any cellular function.

Why does the Tetrahymena respond to hormones of higher organisms?

It seems unlikely that the response of the Tetrahymena to hormones of higher animals were specifically interrelated with any life condition of the unicellular, for the concentration of these hormones in the unicellular's body is low enough to exclude their involvement even in cell-cell communication. Taking into consideration the aquatic existence of the Tetrahymena, it also seems most unlikely that higher aquatic organisms acted on the unicellular through these hormones, since there is no obvious reason for such interaction. A more feasible explanation is that the unicellular's response to hormones of higher organisms forms part of its general response to its environment. There is in fact evidence that the Tetrahymena responds not only to hormones, but also to hormone-like molecules. The plant hormone gibberellin, and the steroid-like molecule benzpyrene have been shown to induce imprinting in the Tetrahymena (21), and non-hormone polypeptides, too, have a similar, although less pronounced effect (22). From this it might be concluded that the Tetrahymena would respond to, and form a "memory" of, all molecules recognized by it as signal molecules, for reasons essential for the survival of its species. However, in this case the receptors presented by the Tetrahymena to higher hormones (or to other signal molecules) are in all probability not preexisting (preformed) structures, but are formed continuously either in absence of the hormone, or specifically in its presence.

Since the recognition of environmental structures is of decisive importance for the unicellular, it seems likely that it

is continuously screening its environment for signal molecules through its dynamically changing membrane patterns (23), and if the latter "recognize" a complementary molecular pattern, the information becomes "fixed" along with the "memory" of the advantageous or noxious nature of the signal received.

Experimental evidence has been accumulating in support of this hypothesis. Tetrahymena cells treated with rabbit serum antibodies to rat hepatocellular membrane receptor (Fig.2)

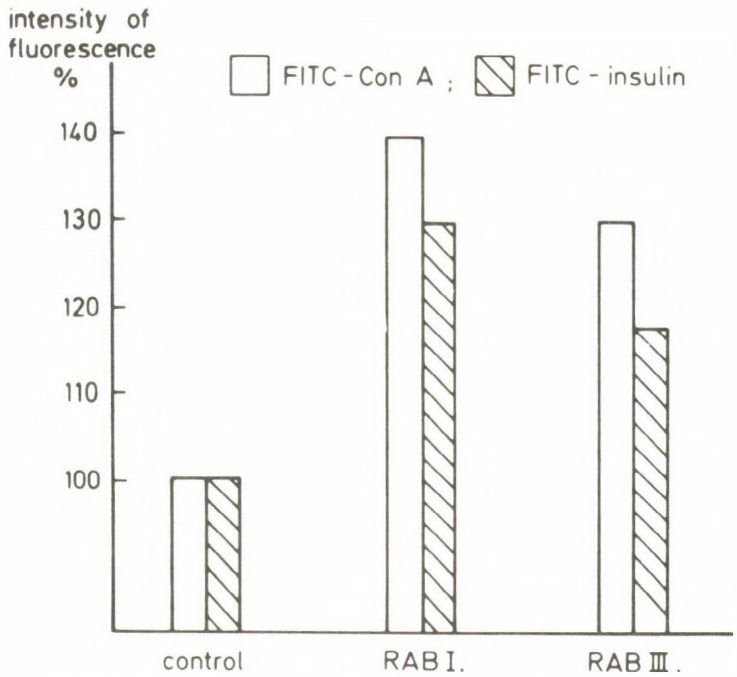

Fig.2.Binding of FITC-Con-A or FITC-insulin by rat liver receptor antibody (RAB-I, RAB-III) treated Tetrahymena related to control as 100.

showed a significantly greater increase in insulin binding capacity than those not treated (24). This has indicated that the antibodies had induced imprinting in the Tetrahymena.The antibodies had been provoked by the hepatocellular receptor, whose molecular configuration is complementary to that of the insulin molecule.It appears that the presence (effect) of the

antibody configuration was sufficient to amplify binding sites for insulin in the Tetrahymena. This supports the implication that not so much the action as the molecular configuration of the hormone played the decisive role in hormonal imprinting. Performance of the experiment on a reverse schedule, i.e. by immunizing the rabbits with Tetrahymena instead of rat hepatocellular receptor (Fig.3), resulted in antibody formation to Tetrahymena (25). Insulin pretreatment of the Tetrahymena cells used for immunization accounted for antibody formation to the insulin receptors as well, to judge from a relative decrease in the insulin binding capacity of rat hepatocellular membrane, compared to the binding relations in presence of antibody stimulated with untreated (control) Tetrahymena cells. The observation that the establishment of imprinting in the Tetrahymena required a certain time implies that certain membrane-associated events have to take place as a prelude to hormone binding. This again suggests that potential binding sites (receptor structures) are not always present in the membrane in a sufficient number; they are disassembled and reassembled continuously until a structure presenting a binding site for the hormone appears, by which the "memory" could be established.

The steroid hormones penetrate the membrane of the Tetrahymena exactly as that of mammalian cells, without, however, binding to the membrane of the unicellular. Nevertheless, pretreatment of the Tetrahymena with a steroid (Fig.4.) induces the formation of cytosolic receptors, which bind the steroid specifically, as demonstrated in isolated cytosolic preparations (26). Thus while binding sites for hormones which interact with membrane receptors may either be present in a low number (or by chance), or easily form, if required, the cytosolic steroid receptors take a relatively long time (several days) to develop into a form transmissible to the offspring generations. Experiments with steroids have shown that these hormones are able to form a receptor for themselves in the Tetrahymena, by induction or probably, by internalization of the initially membrane-bound steroid along with the binding structure (receptor)

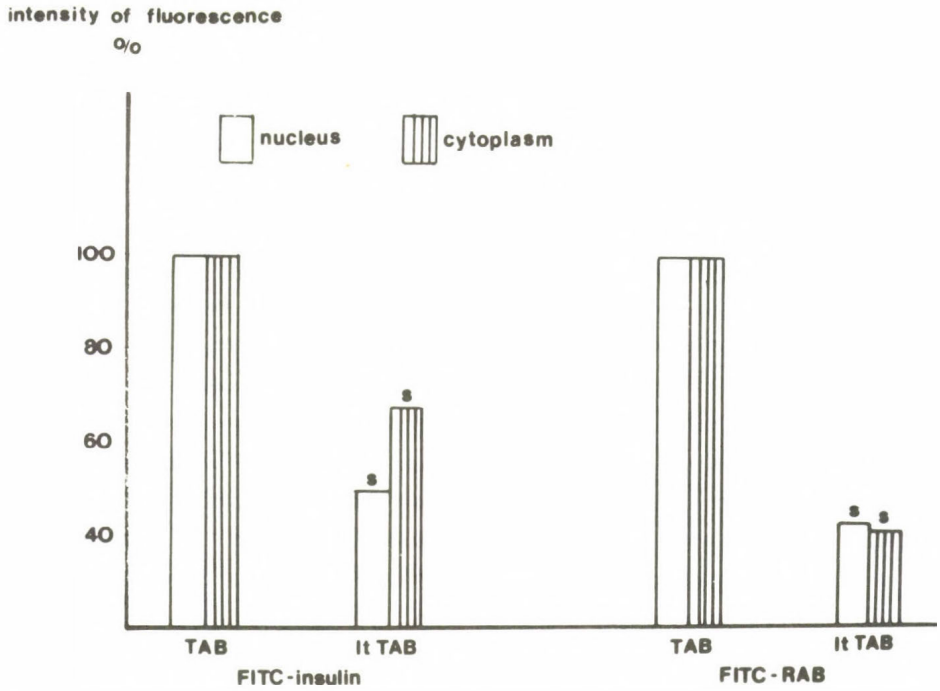

Fig.3.Binding of FITC-insulin or FITC labeled rat liver receptor antibody (FITC-RAB) by rat liver cells pretreated by Tetrahymena antibody (TAB) or insulin treated Tetrahymena antibody (It TAB).

As already noted, preformed receptors interacting with certain materials or types of materials are probably also present in the Tetrahymena, for it obviously needs receptors for its usual nutriments, whose precise recognition is vitally important for its survival. Since these food receptors may well resemble the cellular signal (hormone) receiving structures of higher organisms, they are able to bind hormones either directly, or after transformation to specific binding sites for these. The transformability of food receptors to hormone receptors was suggested earlier by Lenhoff (27) on the basis of his experiments on Hydra, and it may also apply to the Tetrahymena. Fig.5. shows that of the thyroxine series, diiodotyrosine was bound by the Tetrahymena to greater degree than the more iodinated molecules. The basic molecule of the thyroxine series, tyrosine, stimulated the growth of the Tetrahymena of similar degree to diiodotyrosine (Fig.5.) at the

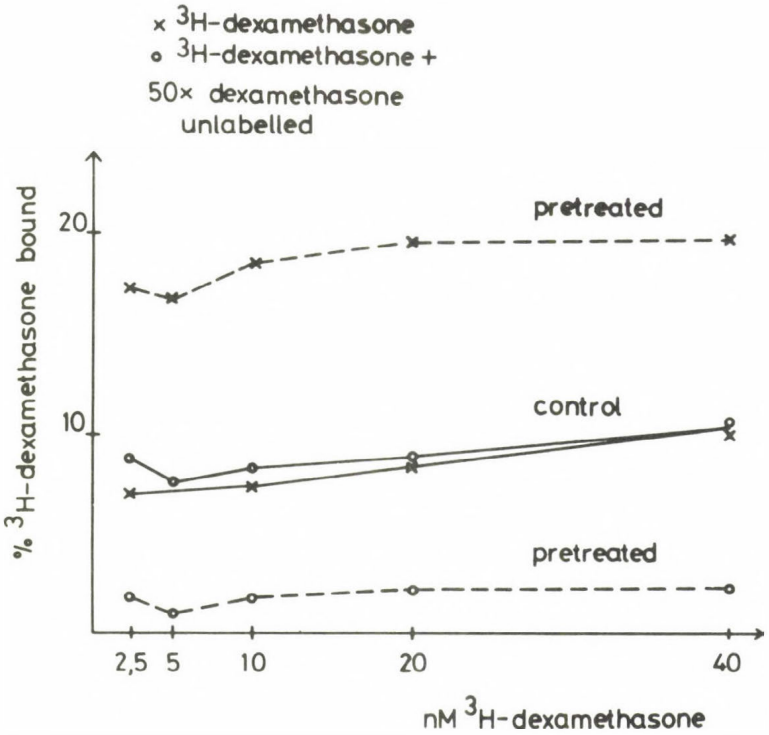

Fig.4. Binding of ^{3}H-dexamethasone by normal or dexamethasone pretreated Tetrahymena in the presence or absence of unlabelled dexamethasone.

same concentration (10^{-8}-10^{-9}M). Thus tyrosine and diiodotyrosine and - in the serotonin series - serotonin and tryptophane bind probably to the same or to similar receptor structures (Fig.6.) Histidine stimulates the phagocytosis of the Tetrahymena of similar degree to histamine, but histamine has no influence on the division of the unicellular. Amplification of the receptor with histidine inhibits histamine action and vice versa, pretreatment with histamine inhibits histidine action (28), owing in all probability to the circumstance that the hormone and the amino acid have a common binding site.

The present experiments have substantiated the implication that receptor formation for hormones of higher organisms is part of the life functions of the Tetrahymena. Precise recogni-

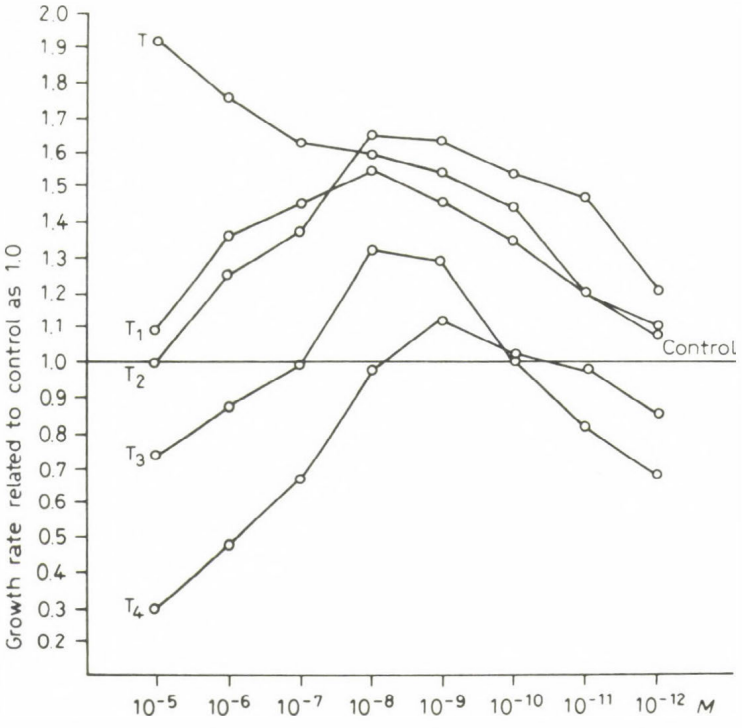

Fig.5.Growth rates of Tetrahymena treated by different concentrations of thyroxine (T_4), triiodothyronine (T_3), diiodotyrosine (T_2) monoiodotyrosine (T_1) or tyrosine (T).

tion of the environment is vitally important for the Tetrahymena and its offsprings - for the survival of the species - and equally important is the "memory" of the signals received. Naturally, the intensity of "memory" varies with the type of the signal received, and depends probably to a greater degree on the actual configuration of the signal molecule than on the potentials of the Tetrahymena itself. While the dynamic membrane is vitally important for the unicellular, certain limitations of membrane responsiveness are equally important at the multicellular level, at which interaction with given hormones requires the presence of specific receptors in given target cells. Encoded in the Tetrahymena is in all probability the dynamic assembly and reassembly of the membrane patterns, whereas in the cells of higher organisms the genetic programme furnishes

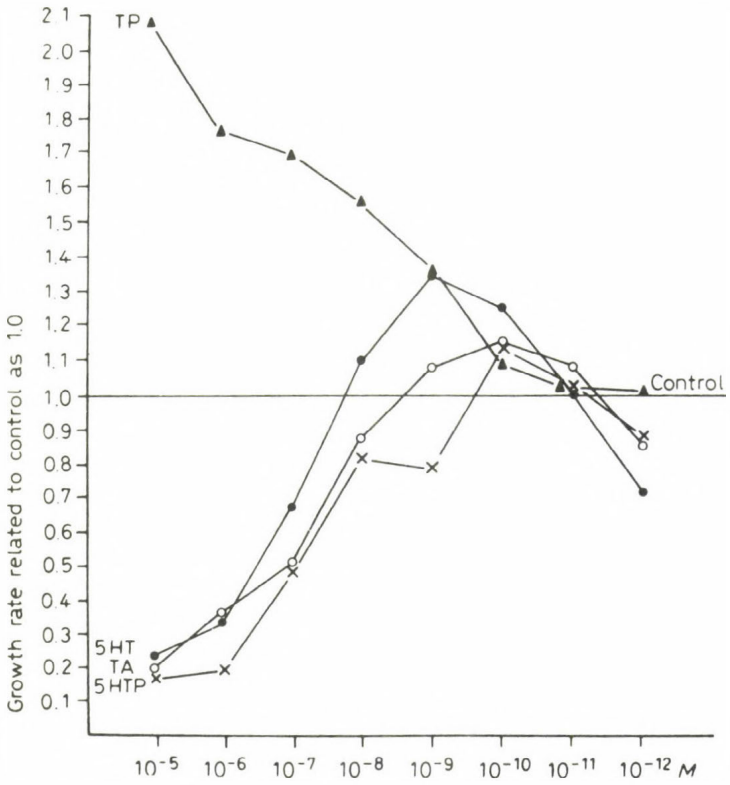

Fig.6.Growth rates of Tetrahymena treated by different concentrations of serotonin (5-HT), 5-hydroxytryptophane (5-HTP), tryptamine (TA), or tryptophane (TP).

the formation of a given number of well defined structures, to ensure homeostasis. For this reason, with the emergence of multicellularity begins the evolution of the receptors which, parallel to the selection of molecules most suitable for the signal function leads gradually to the establishment of lasting receptor - hormone interactions and, ultimately, to the development of the endocrine system (29).

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INFLUENCE OF DECILIATION AND CILIARY REGENERATION ON HORMONAL IMPRINTING IN TETRAHYMENA

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The primary interaction between target cell and hormone gives rise at all levels of phylogenesis to a hormonal imprinting /1,3/ which alters - as a rule increases - cellular response to the hormone, to judge from measurable changes either in the function or in the hormone - binding capacity of the cell on reexposure/s/. The influence of imprinting lasts long, in Tetrahymena over as many as 500 generations /6/. It follows that imprinting is transmitted from one cell generation to the other. Since, however, decrease in the intensity of imprinting is not proportional to the number of generation changes, there is reason to postulate a fixing of imprinting, either at the gene or membrane level. The former hypothesis presupposes gene-level transmission of a membrane - level effect, whereas the latter presumes the transmission of once established receptor structures from mother to daughter cell via self assembly. Since only one part of the membrane of Tetrahymena envelops the cilia, and the other part envelops the cell body, and the first can be relatively easily removed, we relied on this experimental approach to obtain information on the role of the ciliary membrane in the mechanism of hormonal imprinting.

Materials and Methods

Tetrahymena pyriformis GL cells, cultured in 1 % Bacto-tryptone medium containing 0.1 % Bacto yeast-extract / Difco, Michigan, USA / for 2 days at 28°C, were used.

Hormone imprinting before deciliation / A series /:

One mass culture was not treated / control : C /, the other was incubated in presence of 10^{-6} M insulin for 1 hour /insulin-treated: I. Semilente, MC. Novo, Denmark/.

Both cultures were then divided into two groups, of which one was returned to plain medium for 2 days /C, I/, and the other was deciliated by the method of Rosenbaum and Carlson /8/ before return to plain medium for 2 days /C_d, I_d/.

Hormone imprinting after deciliation /B series/:

Part of the cells was deciliated, and placed in plain medium /C_d/. A second cell group was exposed to the above insulin treatment during the first hour of regeneration following upon deciliation /I_1/, wherease the third /I_2/ and fourth /I_3/ groups were insulin-treated after 2 and 3 hours of post-deciliation regeneration, respectively. All cells were then returned to plain medium for 2 days.

After 2 days of culturing in normal conditions, cells of the A and B series were equally fixed in 4% formaldehyde in PBS, and incubated for 1 hour in presence of FITC-labeled insulin /protein concentration: 0.5 mg/ml; FITC/ protein ratio: 0.12/ after washing in three changes of PBS. Then cells were washed again, spread on slides, dried, and assayed for intensity of fluorescence with a Zeiss fluoval cytofluorimeter, connected with a HP 41/C calculator for automatic analysis of intergroup differences for significance by Student's t test, and analysis of variance. 25 cells were assayed for fluorescence in each group, and each experiment was performed in four replicates. The mean values assessed in the experimental groups were related to the FITC-labeled insulin binding of the control cells /C, C_d/ as 1.00.

Assessment of the Return of Cell Motility

The deciliated cells were suspended in 20 vol. of medium, and trypan-blue was added to the suspension to identify the

dead cells. Moving cells were counted under the microscope every 15 min. Six replica experiments were performed.

Results and Discussion

The cytoplasmic membrane of Tetrahymena envelops partly the cilia. Thus deciliation involves a considerable loss of membrane and, since the ciliary membrane plays a leading role in ligand binding /5/, a still greater loss of membrane receptors. It follows that deciliation after primary exposure to insulin deprived Tetrahymena of a considerable part of its insulin-amplified /imprinted/ receptors in a very early period of imprinting. Regeneration for 2 days in plain medium was nevertheless sufficient to compensate for the greater part of receptor loss, to judge from the observation that the progeny cells of those deciliated after imprinting did not bind significantly less insulin than those not deciliated after preexposure /Fig. 1 /.

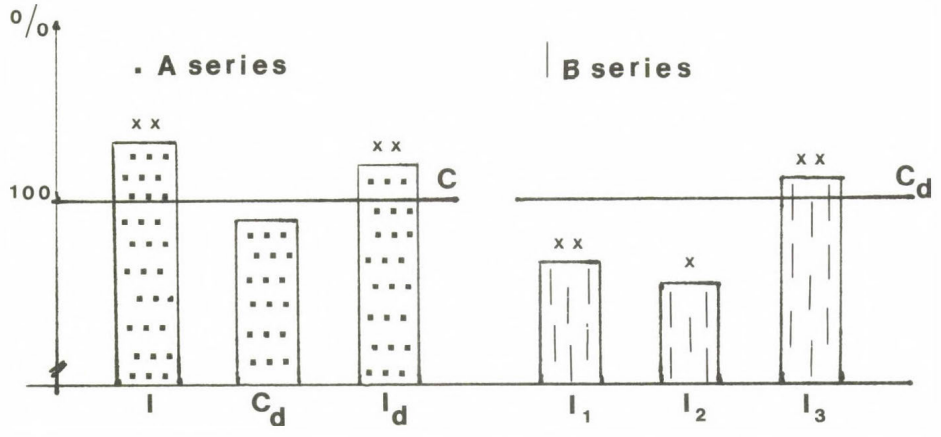


Fig. 1. Binding of insulin to control /C/, deciliated /C_d, I_d/ insulin-treated /I/ and regenerating cells /I_1, I_2, I_3/ 1,2 and 3 h after deciliation. Significance related to control: xx $p<0.01$; x $0.05>p>0.02$.

During ciliary regeneration the membrane phospholipids are derived from subsurface vesicles /7, 11/ and the membrane proteins migrate into the ciliary membrane region from the inner membrane envelope by lateral diffusion /9, 10/. Certain authors have postulated that the latter proteins are rapidly incorporated into the subsurface vesicles, and merge with the outer membrane along with lipids, thereby promoting its regeneration /10/. Whichever hypothesis holds, ciliary membrane regeneration seems to restore the pre-deciliation pattern, including the details characteristic of imprinting.

Functional regeneration essentially corresponded to the morphological picture, for post-deciliation moving cell count took 3 h to approximate the original value /Fig. 2/.

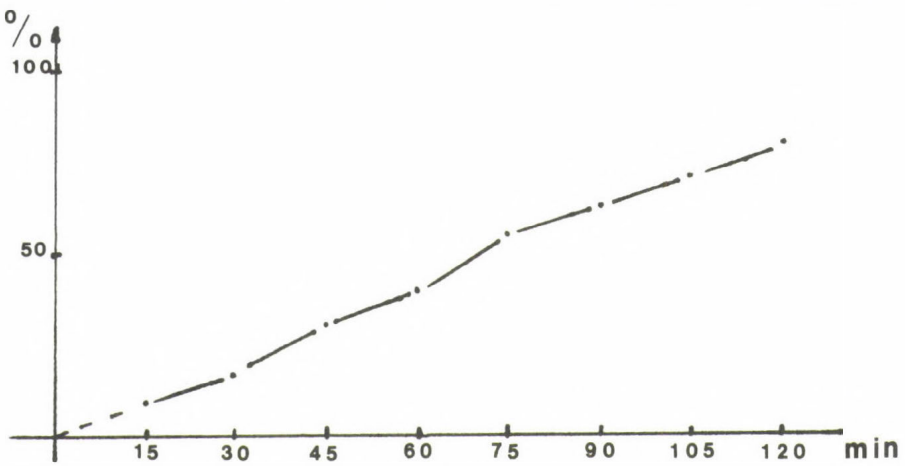
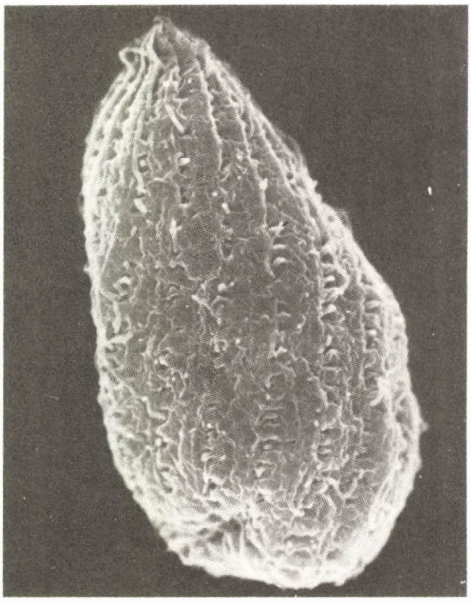
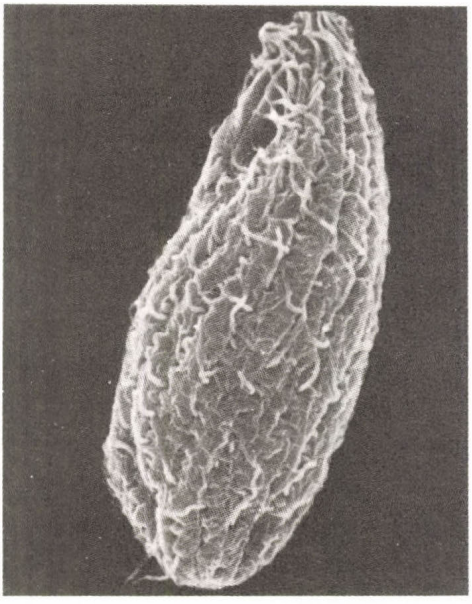


Fig. 2. Proportion of moving cells during the first 2 h after deciliation. Peak, approximating original value is reached about 1 h later.

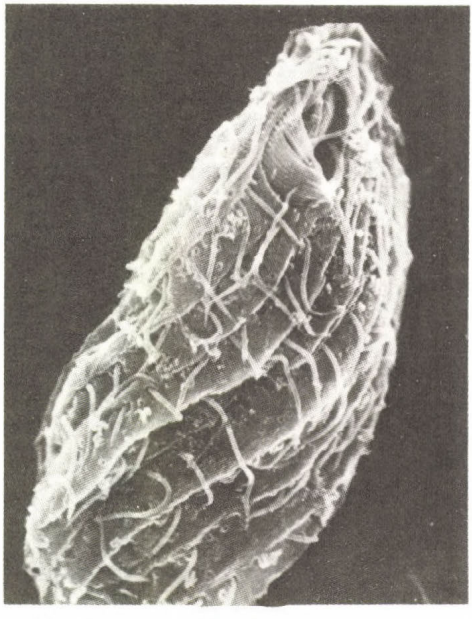
Scanning electron micrographs show that deciliation by Rosenbaum and Carlson's method /8/ had been complete, with the exception of the oral field. Rudimentary cilia re-appear after 40 min. Full-length cilia began to appear after 2 h, in a much lesser number than usual. Regeneration was completed by 3 h /Figs 3, 4, 5, 6/.



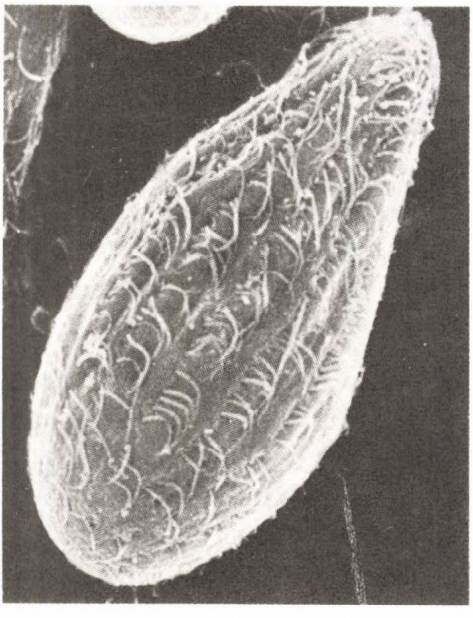
3 . x 5100



4 . x 4500



5 . x 6000



6 . x 4000

Figs 3, 4, 5, 6 : Scanning electron micrographs show the cell after deciliation /3/, after 40 min regeneration /4/, and after 120 min or 180 min regeneration /5, 6/.

The cilia were undeveloped at 1 and 2 h after deciliation and, as indicated by the results, imprinting by insulin failed to take effect at those points of time, whereas it did take place, even if to a somewhat lesser degree, on exposure to insulin 3 h after deciliation.

It follows that intact cilia are indispensable for hormonal imprinting in Tetrahymena but, as indicated by the reexposure studies, deciliation does not abolish imprinting once it has been established.

Involvement of the cilia in the mechanism of imprinting is thus an established fact, while the ciliary localization of recirculated imprinting remains obscure. One may speculate that during the 1 h of insulin exposure which preceded deciliation, the hormone molecules may well have become internalized and, unless degraded intracellularly, may have forwarded the information to the nucleus. In this light fixing of imprinting at membrane or nuclear level is equally possible. A third alternative is that the subsurface vesicles arise by membrane internalization /10/, and therefore carry all membrane-associated information including hormonal imprinting, in a form suitable for recirculation. However, the mechanism by which information is recirculated into the regenerated cilia remains obscure until the mechanism of imprinting itself is eluciated.

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Symposia Biologica Hungarica 33 (1986)

DRUG INDUCED MEMBRANE PERTURBATION IN TETRAHYMENA PYRIFORMIS. STUDIES ON LECTIN BINDING

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Cells are covered by membranes, their receptors and marker structures have an important role in the complexity of the cell and the environmental factors. Dynamic changes of these behavioural data make the membrane possible to rapid structure-correction. On the other hand such important structural characteristics are also present conservation of which is the basic condition of living cell. This "conservative" plastic membrane is the essential element of integrity of both the unicellular and multicellular organisms. This integrity may be influenced by: 1/the internal regulation of cellular life-processes and 2/ the external milieu.

The unicellular organisms are very sensitive to changes of the behavioural conditions. This is the case in our model object, the Tetrahymena pyriformis, too. /2/.

Different drugs can modify the membrane by reacting with the membrane or the associated enzymes. These perturbations are well characterized by lectin binding because of the structural changes of the membrane alter the position or density of several saccharides, particularly the organization or composition change of these molecules in the unit-membrane. Temperature alterations also transform the membrane composition. Long-term heat exposure decreases the fluidity, while the cold-shock renders it more fluid /5/.

In the following we shall describe the effects of these two different environmental factors - drugs and temperature changes -, how they can modify the membrane structure, especially the orientation or number of saccharides in it.

Phenothiazines like trifluoperazine, propericiazine and chlorpromazine or some local anesthetics - dibucaine, tetracaine, lidocaine and procaine - are able to perturb the membrane composition by way of inhibition of membrane associated guanylate cyclase /4/. We could illustrate the result of these effects by the binding profile of applied lectins.

After phenothiazine treatment we can register a special binding for certain lectins /Fig.1/. Maximal lectin binding was observed for Phaseolus and Helix lectins with the treatment of all kinds of phenothiazines. The ligand of these two lectins is the same saccharide, N-acetyl-galactosamine.

Minimal binding differences depend on the type of phenothiazine molecule. In the case of trifluoperazine and propericiazine the Datura, in the case of chlorpromazine the Concanavalin A and the Datura presented the lowest level.

Local anesthetics produced agreeing and discordant data, too. /Fig.2/. By the influence of dibucaine three lectins: Datura, Phaseolus and Helix represented the maximal lectin binding, while the other pole was presented in the Lens lectin. Action of tetracaine /Fig.3/ results a sinking of all the bindings, Helix presents the minimal point and Lens the maximal point of this profile.

In the lidocaine treated cells /Fig.4/ the decrease of the lectin binding was the most explicit. Only the Lens binding values elevate over the control level - this is the maximum point-, the lowest binding has been shown by Datura and Helix. The group of procaine treated cells /Fig.5/ was the only where the binding of Phaseolus and Helix opposed, Phaseolus has the maximal Helix the minimal values.

If we compare these results to the thyrotropic hormon /TSH/ binding values of TSH treated - imprinted /1/ - and certain phenothiazine or local anesthetics treated cells /4/, the increase and decrease seems to be parallel with lectin binding profile of Phaseolus and Helix lectins. Both ligands are glycoproteins, N-acetyl-galactosamine molecules. Since the TSH-receptor contains a ganglioside-group, it seems to be possible that the ganglioside is just the size G_{M2}, containing also a N-acetyl-galactosamine in terminal position.

The drug-effects investigated above are specific, enzyme-linked membrane perturbations. We can demonstrate this by ethanol treatment, which also perturbs the membrane, but by another mechanism /Fig.6/.

Fig.1 Lectin binding of Trifluoperazine ▨ Propericiazine ▥
and Chlorpromazine ▨ treated Tetrahymena cells

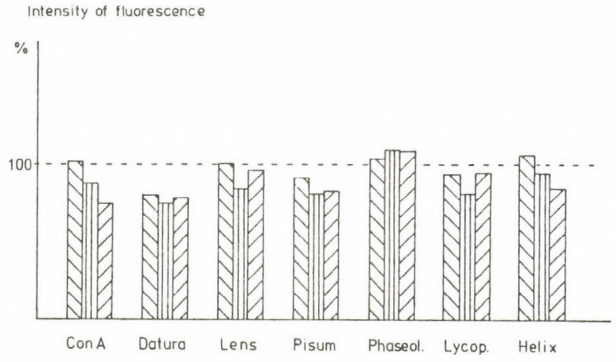
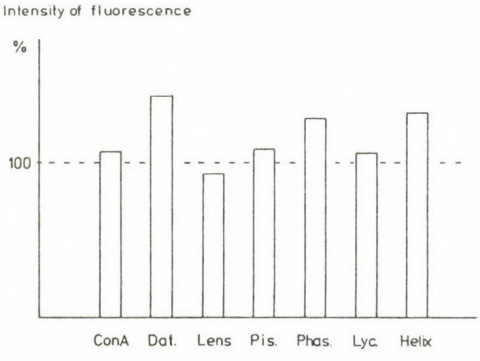


Fig.2 Lectin binding of Dibucaine treated Tetrahymena cells

Fig.3 Lectin binding of Tetracaine treated Tetrahymena cells



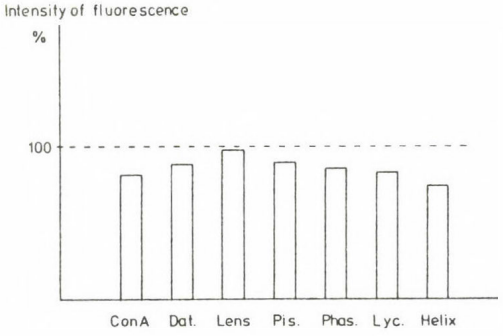
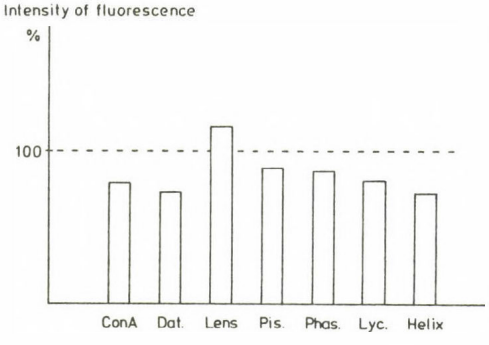


Fig.4 Lectin binding of Lidocaine treated Tetrahymena cells

Fig.5 Lectin binding of Procaine treated Tetrahymena cells



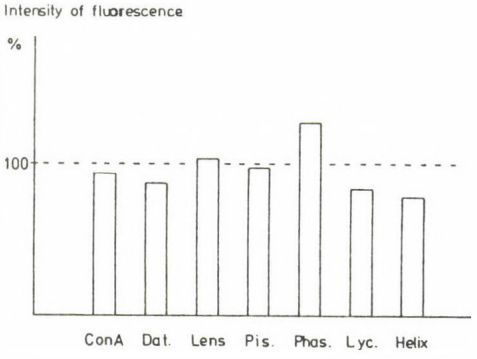


Fig.6 Lectin binding of Ethanol treated Tetrahymena cells

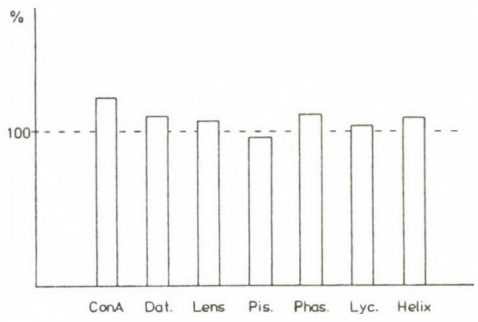


Fig.7 Effect of long-term cold-shock (15 °C) on lectin binding of Tetrahymena cells

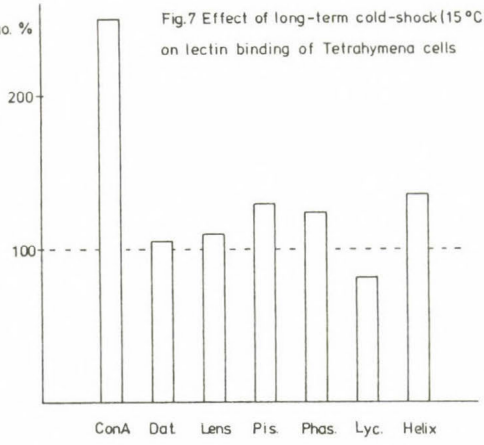


Fig.8 Effect of short-term cold-shock (28 °C→15 °C) on lectin binding of Tetrahymena cells

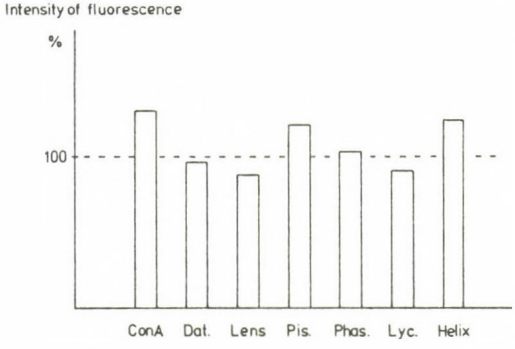


Fig.9 Effect of short-term heat-shock (15 °C→28 °C) on lectin binding of Tetrahymena cells

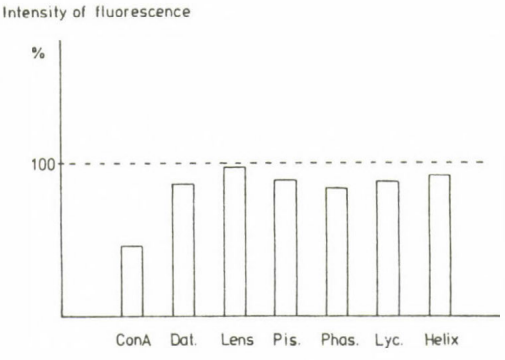


Fig.10 Effects of long-term cold-shock (15 °C) and Ergosterol treatment on lectin binding of Tetrahymena cells

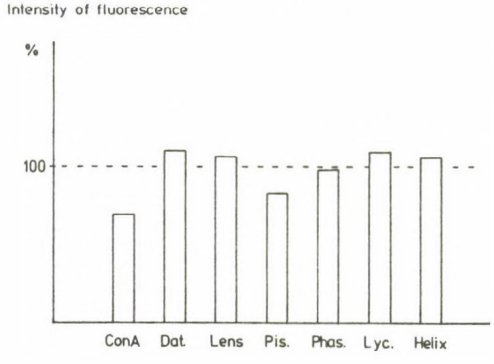
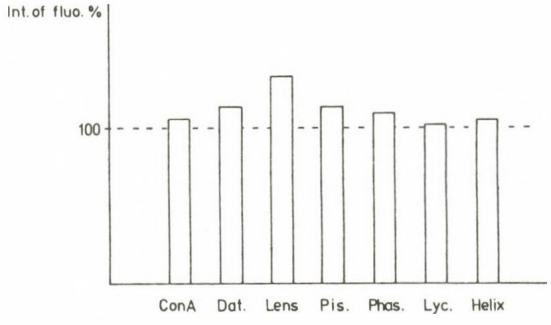


Fig.11 Effects of short-term heat-shock (15 °C→28 °C) and Ergosterol treatment on lectin binding of Tetrahymena cells



In this case the Concanavalin A presents the highest binding level indicating that ethanol treated membrane is abundant in D-glucose and D-mannose molecules, which are able to bind this lectin.

In further experiment we observed the altered lipid-phospholipid composition of membrane influenced by ergosterol treatment or temperature changes. These membrane alterations were studied also with the help of lectins.

Ergosterol treatment induces a new, rigid membrane in the following ways: 1/ indirectly - helps the new neutral lipids to build in the membrane; and 2/ directly - when the ergosterol molecules themselves change places with tetrahymenol, the special lipid of Tetrahymena /3/. Temperature adaptations are also able to transform the organization of membrane structure: warm environment increases its fluidity, in cold conditions it becomes more rigid. But these changes are transient, after certain time the cell adapts to the shift temperature by modifying the structure of membranes. Finally, the result of these modifications is that warm conditions increase the rigidity, and the cold culturing the fluidity of the membrane to its original state. The reason of this is the flow of microsomal lipids towards the cell surface. Between these two effects /the ergosterol and temperature impression/ there was a relation in respect of membrane fluidity and structure, considering their saccharide components.

The permanent or the transient effect of cold /Fig.7-8/ resulting a more fluid membrane, increases the binding of Concanavalin A, Pisum and the Phaseolus and Helix lectins, presumably by changing the organization of glucose, mannose and their derivates as well as N-acetyl-galactosamine in the membrane.

The thermo-shock /Fig.9/ - heating - causes the opposed alterations of saccharide composition according to lectin binding.

The effect of ergosterol /Fig.10-11/ stiffening of membrane depends also on the other parameters, like temperature. The main result is, nevertheless, the high level of Datura, Lens and Lycopersicon lectin binding and consequently the increasing of N-acetyl-glucosamine molecules in this membrane.

All these results fortify our opinion that the environmental factors have a special importance in the determination of membrane-processes.

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ADVANCES IN FREE-LIVING PROTOZOA RESEARCH

II. ECOLOGY, WATER AND SOIL

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USE OF PROTOZOAN COMMUNITIES IN PROTECTING AQUATIC ECOSYSTEMS

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ABSTRACT

Microbial species play important roles in aquatic ecosystems by fixing energy in photosynthesis and by processing dead organic material and associated bacterial flora. Protozoan species span the range of sensitivities for other taxonomic groups and can be manipulated easily under laboratory conditions to determine community-level responses to ecosystem stresses. Artificial substrates allow statistical evaluation of replicate microecosystems. For example, protozoan colonization experiments carried out in the laboratory show that protozoan communities respond to toxic materials such as cadmium and copper at levels near the limits of analytic precision and in the same range as estimates of no-effect concentrations derived from large single species data bases. Protozoan communities can be obtained from particular ecosystems of interest (or from standard or pristine ecosystems) and tested for their response to toxic materials. Predictions based on microbial tests using these communities can be validated directly in receiving ecosystems using essentially the same techniques as in laboratory tests. Testing of microbial community dynamics can provide direct estimates of thresholds and breakpoints in ecosystem responses to stresses. Such responses can be determined over a comparatively short period of time (a few weeks) as compared to predictions based on extrapolations from single species data that must be collected over a period of several months to several years.

INTRODUCTION

Man's effect on natural ecosystems has shifted from the use of natural resources as free goods toward the management of ecosystem services. This has become necessary because unmanaged ecosystems heavily impacted by human

activities stop providing necessary goods and services. Deplorable conditions in many ecosystems have reversed after lessening or removal of exogenous stress. Human societies must learn to manage ecosystems for necessary services (such as low levels of waste disposal and heat dissipation) without degrading these systems such that ecosystem services are lost or adverse human health effects arise.

What role do microbial communities play in maintenance and management of ecosystems? Much human impact on natural ecosystems occurs in rivers and nearshore areas of lakes and seas. Shallow-water biota are often the first to be affected, often severely. Shallow-water ecosystems are dominated by microbiota, such as Protozoa, algae, and bacteria, that play major roles in primary production and degradation of dead organic matter. These commonly impacted biotas are logical choices for evaluating potentially adverse human effects. Microbial communities form the base of food chains leading to more familiar species and can often provide answers to important questions more rapidly at lower cost than if higher taxa were studied. This paper details some of the ways we have used Protozoa in evaluating and protecting freshwater ecosystems.

Protozoan communities provide a great deal of information relevant to assessing human impacts on complex biological systems. Levels of biological organization above the single species (population) have important characteristics (e.g., predation, succession, competition) that are not considered in most single species testing schemes (Webster 1979, Cairns 1983). Protozoa have a number of characteristics that make them ideal test organisms for evaluating potential human impacts, ecosystem damage, and recovery. These characteristics include cosmopolitan distribution of most species, predictable colonization of artificial substrates by Protozoa, tolerance of natural communities to the laboratory, and sensitivity of Protozoa to toxic and nutrient inputs. Sampling using artificial substrates also provides a practical means of comparing field and laboratory results, increasing the probability that laboratory predictions will be validated in actual receiving ecosystems.

COSMOPOLITAN DISTRIBUTION

When the cosmopolitan distribution of protozoans is discussed, it is frequently noted that protozoans do appear to be quite similar structurally all over the world. The number and placement of cirri on a hypotrich, for example, appear to be remarkably consistent in Europe, North and South

America, Australia, New Zealand, Asia, and Africa. Opponents of the cosmopolitan distribution hypothesis contend that structural characteristics may be similar throughout the world but, behind this structural facade, is an array of remarkably different physiological species. The most common illustration of this is the mating system in Paramecium where each mating type might reasonably be considered a separate species. Demonstrations of physiological species sharing a common structural facade are not abundant, but this might be due to the level of research effort directed toward confirming this hypothesis rather than to the fact that Paramecium is the exception to the rule.

Since it is unlikely that this situation will quickly be resolved, one might ask the question: "what difference will these physiological species make in carrying out ecological studies?" Physiological races or sub-species are well known for terrestrial plants and certain terrestrial animals and are well established for a variety of aquatic organisms. While these invisible (i.e., non-structural) attributes may make the interpretation of results more difficult, they do not seem to have seriously impaired investigators carrying out ecological studies.

ARTIFICIAL SUBSTRATES

The MacArthur-Wilson (1967) equilibrium model represented a major conceptual advance in ecology. Although it was developed from observations of bird colonization of oceanic islands, it was first confirmed in an experimental way with protozoans (Cairns et al. 1969). A substantial number of papers, some supporting and some critical, have appeared since the equilibrium model was first published. A discussion of this substantial literature is beyond the scope of this manuscript. It is worth noting that the model is useful in explaining freshwater protozoan colonization processes, even though it was developed for another, quite distinct taxonomic group. Repeated verifications of the usefulness of colonization assessments are extensive and include experiments conducted in the field (e.g., Henebry and Cairns 1984, Plafkin et al. 1980) and the laboratory (Cairns et al. 1980, Pratt and Cairns 1985a, Niederlehner et al. 1985).

COMMUNITY-LEVEL TOXICITY TESTING

A major problem associated with toxicity testing of aquatic organisms is obtaining sufficient data on which to base predictions of environmental safety and harm. If the sensitivity of species to a toxic chemical is

normally distributed, it should be possible to estimate a level that would adversely affect only a small proportion of extant species. The traditional approach has been to conduct a series of single species toxicity tests with an array of test populations and to estimate a safe level by multiplying some acute or chronic toxicity level of a sensitive species by an application factor (commonly 0.1 or 0.01) to account for extreme sensitivities of untested organisms. Such procedures ignore the fact that populations in nature do not exist in isolation, that biological interactions (e.g., predation, competition) may be important in determining environmental responses, and that toxic chemicals rarely occur singly in effluent streams. Different responses of test populations of the same species under different abiotic conditions further complicates analysis of single species tests, even though such tests can have a high degree of replicability and precision. Many tests use organisms that may never be found in the actual receiving system impacted by tested chemicals.

An alternative approach is to estimate safe levels directly using microcosms or community-level tests. By removing and manipulating a subset of a natural community, it is possible to obtain a greater degree of environmental realism. We have used protozoan communities developed on artificial substrates as model systems. Standard test systems have been proposed (Cairns et al. 1985, Niederlehner et al. 1985). Such microcosms test a large number of species simultaneously. Many natural processes continue in the laboratory, and diverse communities can be maintained for extended periods under controlled conditions (Pratt and Cairns 1985a). Properly developed microcosms can be replicated, and important system-level characteristics can be tested using complex communities (e.g., Giddings 1983).

We have examined the response of protozoan colonization to toxic compounds in laboratory microcosms. We hypothesized that a large array of protozoan species (40-80) would have a range of sensitivities to toxic materials and that adverse effects on certain species could be measured as reductions in colonizing species in direct response to the concentration of toxic material. Protozoans and other microbes have tolerance ranges similar to other taxa (Patrick et al. 1968, Ruthven and Cairns 1973), so it is reasonable to expect that safe concentrations for these organisms might be safe concentrations for higher organisms. Protozoans are especially attractive for such experiments because they are readily available at low cost anywhere in the world; they may be collected from a receiving system of interest without damaging the indigenous populations; they can be tested in small containers; they

have short life cycles so that generational tests are possible in hours or days; their surface to volume ratio is high, ensuring intimate contact between the organism and the test material; and they represent a significant portion of the biomass of aquatic ecosystems. Under certain circumstances, culturing in well-defined media may be helpful despite the fact that the environmental realism of such tests may be low.

Our experiments have shown that colonization of initially barren polyurethane foam (PF) artificial substrates by Protozoa in the presence of heavy metals yields estimates of adverse effects comparable to estimates from large data bases of single species test results. In these experiments, PF artificial substrates were colonized to species equilibrium in an unstressed natural ecosystem and then used as species sources for colonization of initially sterile artificial substrates in laboratory microcosms dosed with toxic materials. For example, Niederlehner et al. (1985) estimated the chronic effect level for cadmium based on impairment of colonization at 0.8 µg/L, within the range of chronic effect levels for other taxa (0.08-11 µg/L) and below the current criterion of 2.48 µg/L (United States Environmental Protection Agency 1980). Studies of complex effluents containing heavy metals showed impairment of colonization (Fig. 1) at levels corresponding to dissolved copper concentrations of 14 µg/L, near the present

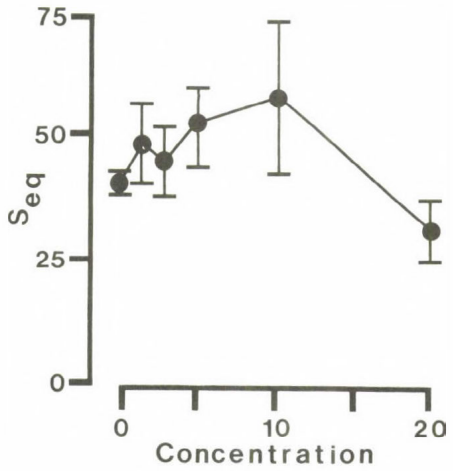

Fig. 1. Effect of a complex effluent containing copper on protozoan colonization in laboratory microcosms. Ordinate is estimated equilibrium species number (S_{eq}); abscissa is effluent concentration in percent (20% effluent = ~14 µg/L).

criterion of 6.82 µg/L. Lower concentrations had no adverse effect on experimental microcosms. Over the 21-28 day course of these tests, we saw no evidence of direct toxic effects on species sources.

ECOSYSTEM ASSESSMENT

Protozoan communities have been useful in examining the condition of ecosystems in several ways. Much use has been made of traditional indicator species (e.g., Sladecek 1973). Natural and artificial substrate communities have also been used in more traditional ecosystem surveys of stations along a stream. Higher level statistical analyses now permit more extensive analyses of complex species data sets. Recently, protozoan communities have been used to assess the recovery of damaged ecosystems and to validate laboratory predictions under real ecosystem conditions.

Cairns (1949) showed that, in addition to their traditional value as indicator species of organic enrichment, the structure of protozoan communities could be used to assess a variety of other types of pollution, including industrial. The colonization pattern of protozoans on artificial substrates placed in natural systems was also a superb means of determining degree of eutrophication (Cairns et al. 1979). More recently, Pratt and Cairns (1985b) showed that distribution of species into functional groups is quite orderly and may be a useful new measure of aquatic ecosystem condition. One of the major advantages of functional groupings is that less taxonomic skill may be necessary for analysis than when each taxon is identified to species. Recently, Pratt and Cairns (1985c) have summarized the overall value of Protozoa as ecological indicators.

One important question is how much stress an aquatic ecosystem assimilates without being placed into disequilibrium or altered significantly in structure and function. An example of how protozoans may be used to estimate assimilative capacity may be found in Hart and Cairns (1984). Early colonization patterns of diatoms and protozoans are quite different (Cairns et al. 1983, Stewart et al. in press). As a consequence, each group of microorganisms furnishes different information and, therefore, other groups should also be studied rather than selecting a single group to represent the entire microbial community. One of the questions for regulators and for society in general is the cost effectiveness of the information gathered on the condition of aquatic ecosystems. The Academy of Natural Sciences of Philadelphia (1984) found that in the Flint River/Lake Blackshear ecosystem, protozoan community analysis provided the best biological correlate of physico-

chemical differences at a cost far below that of other major groups of aquatic organisms.

In recent studies of reclamation of lakes following phosphate mining in Central Florida, we found that protozoan colonization of artificial substrates was a useful measure of ecosystem condition. Protozoan fauna in reclaimed ecosystems developed quickly toward that found in natural ecosystems in the same geographic area (Pratt et al. 1985). In these studies, lakes of differing age were examined and compared for their protozoan colonization dynamics and physico-chemical differences. Newly reclaimed lakes (<2 years old) had depauperate faunas (Fig. 2), depressed pH (~4.5), and high levels of dissolved aluminum and selenium. These differences disappeared in lakes >2 years of age. Community analysis (detrended correspondence analysis) failed to detect significant differences among communities from lakes >2 years old. Differences among microbial communities in differing ecosystems are the result of differential dispersal and the appropriateness of conditions encountered by new colonists.

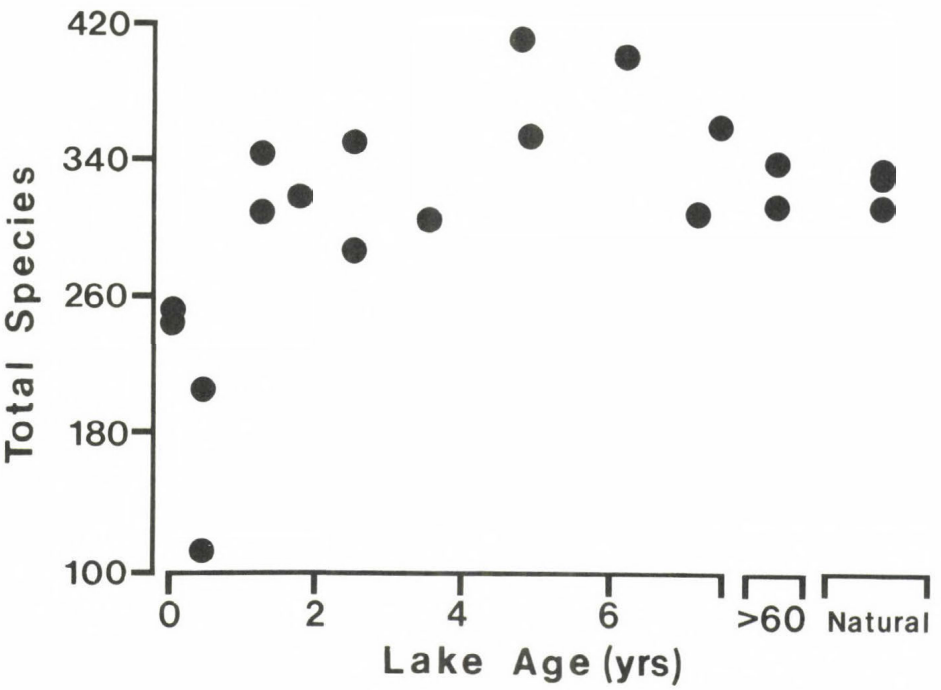

Fig. 2. Total protozoan species collected on artificial substrates in recovering phosphate mine pits and natural lakes in Central Florida, USA.

Direct prediction of ecosystem effects from laboratory tests is rare. Recent studies by Shen et al. (1985) have shown that effects in laboratory colonization experiments are directly verifiable in the field. She and her colleagues compared protozoan colonization in laboratory systems dosed with complex effluent and found excellent correspondence to colonization in an adversely impacted receiving system. These experiments suggest it should be possible to predict adverse effects, recovery zones, and no effect levels based on simple laboratory analyses of effects on protozoan communities and to validate these predictions using study methods comparable to those used in the laboratory.

FUTURE NEEDS

Much information will be necessary to verify the use of protozoan testing as a worthwhile alternative in assessing potential human effects on ecosystems. Evaluation of the response in laboratory testing systems to an array of toxicants is essential. Understanding the degree to which physiological differences among structurally identical protozoan species affects the outcome of ecological and toxicological studies is the single most important question confronting protozoan ecologists today. Further demonstration of the ability to predict adverse ecological effects and validate these predictions is essential.

Simple, direct methods are necessary to predict effects of increasing human influence on the biosphere. It is clear that testing of protozoan communities may play a key role in developing environmentally realistic, cost effective methods for such evaluations. The tests may have greater transferance to other nations and may be less subject to criticism than tests using geographically distinct faunas. Such a test has been proposed as a global standard test (Cairns 1985). It should be possible to select several type ecosystems against which to reference testing results and compare site-specific responses, since Protozoa are represented in every ecosystem. Whole communities demonstrate a wealth of important ecological responses not testable using common and traditional toxicological methods (Cairns 1979, 1983). Ecologists will be expected to develop better predictors of environmental effect, and protozoan tests can provide valuable predictive information.

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THE INDICATOR VALUE OF FREE-LIVING PROTOZOA

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In Czechoslovakia, standards for drinking water allow for the presence of up to 20 individuals per ml in communal water supplies, and up to 100 colourless flagellates and 10 ciliates per ml in local supplies. The identity of the organisms is not considered. By noting only the number of individuals present, it is quite possible to exclude information about the quality of the water which could be obtained from identifying organisms to genus and species. The absence of information on the identity of the protozoa does present problems in employing standards for biological evaluation. One problem is that species which are characteristic of polluted surface waters are found in clean waters underground. Common types of colourless flagellates include species of the genera: Bodo, Cyathomonas and Cercobodo. Among the ciliate genera more frequently represented are : Cyclidium, Mesodinium, Microthorax, Paramecium and Spirostomum. These genera of flagellates and ciliates are characteristically associated with organic matter which may be formed by surface autotrophic activity and percolated into ground water from polluted surface water. It is also possible that the species derive from run-off from cultivated fields. The low quality of the water may be confirmed by microbiological analyses which reveal large numbers of coliform bacteria. Chemical analyses often are unable to indicate any problems because of the low concentrations of pollutant.

The presence and identity of protozoa in underground water is held to be of indicative value. Up to now their value

has not been fully recognized and they have been underexploited in this area. In long-term pumping tests quality control by chemical analyses was always carried out, sometimes microbiological analyses were done but never any biological ones. Now we amplify biological evaluation not only for shallow subsurface waters but also for deep waters up to 300 m below the surface. This form of analysis is also carried out to assess the impact of fissures or breaks in the bedrock through which pollution might penetrate to the water bearing layers.

A poor quality of water was often found in wells and bore holes from which water was pumped at irregular intervals. Unsuitable waters were less common in continually pumped wells. However, excessive exploitation of ground water can lead to the penetration of pollutant from the surface. This is because excessive pumping causes the ground water level to drop significantly and thus increase the gradient from surface to ground water. The quality of the water drawn-up at regular pumping of minimal amount of water must be maintained as well, otherwise casings of wells become clogged. This will lead to a decline in water supply and in the water quality. The decline in quality results from the stagnation of water caused by the low amount of water flow. This situation is referred to as "self-pollution" and is associated with the appearance of species of protozoa connected with poor water quality.

The appearance of protozoa in underground water reflects the world-wide increase in pollution. It is important that we collect appropriate ecological data. The properties of the ground water may be expressed by a saprobic index, just as surface water. The application of saprobic indices developed from studies of surface water to ground water is not straightforward because they have, until now, not included these species occurring in ground water. Most previously recognized indicator species were associated with polluted surface water, sewage and sewage treatment. In addition, it is hard to think that one species which occurs in polluted surface water but clean ground water has the same indicative value. With Prof.Dr.Sládeček,Dr.Sc. we have attempted to provide

saprobity values as to correct existing ones (Sládeček et al.1981, Moravcová 1977,1978). Some protozoa have no saprobic index as they are rare in underground waters and no comparisons can be made.

For the reasons given above, I would like to ask colleagues working with free-living protozoa to join us in the search for more ecological information. With such information it might be possible to compare and revise existing indices. This is an urgent and necessary task if we are to use biological analysis of underground water quality.

In conclusion, I would like to mention some other ways we might use the indicative value of protozoa. They could be used to identify sources of water seepage in the vicinity of dams and reservoirs used for drinking or for industry; to assess the different stages of water treatment; for deciding if water, after washing in rapid sand filters, should be directed to a reservoir or to sewarage. Protozoa may also be used in artificial recharge trials. Glass slide method of water quality evaluation (Sládečková 1972, Moravcová 1981) may be used in wells or bore holes and even of water removed by indirect withdrawal from gravel pit reservoirs. (In indirect withdrawal, the water percolates through gravel and sand layers to bore holes in the banks, Moravcová et al. 1974.)

List of colourless flagellates found in underground water

Anisonema ovale KLEBS
Bikosoeca planctonica KISELEV
Bodo angustus (DUJ.)BÜTSCHLI
Bodo edax KLEBS
Bodo fusiformis (STOKES)LEMMERMANN
Bodo globosus STEIN
Bodo lens (MÜLLER) KLEBS
Bodo minimus KLEBS
Bodo obovatus LEMMERMANN
Bodo repens KLEBS
Bodo uncinatus (KENT) KLEBS
Cercobodo agilis (MOROFF) LEMMERMANN

Cercobodo bodo (MEYER) LEMMERMANN
Cercobodo grandis (MASKELL) LEMMERMANN
Cercobodo longicauda (STEIN) SENN
Cercobodo ovatus (KLEBS) LEMMERMANN
Cercobodo simplex (MOROFF) LEMMERMANN
Collodictyon triciliatum CARTER
Cyathomonas truncata (FRES.) FROMENTEL
Entosiphon obliquum KLEBS
Entosiphon ovatum STOKES
Euglenopsis vorax KLEBS
Hexamitus pusillus KLEBS
Menoidium incurvum (FRES.) KLEBS
Monas arhabdomonas (FISCH.) H.MEYER
Monas minima H. MEYER
Oicomonas termo (EHR.) KENT
Peranema trichophorum (EHR.)STEIN
Petalomonas angusta (KLEBS) LEMMERMANN
Physomonas vestita STOKES
Pleuromonas jaculans PERTY
Rhynchomonas nasuta (STOKES) KLEBS

List of ciliates found in underground water

Chaenea limicola LAUTERBORN
Chaenea vorax QUENNERSTEDT
Chilodonella cucullulus O.F.MÜLLER
Chilodonella uncinata EHRENBERG
Cinetochilum margaritaceum PERTY
Colpidium colpoda (EHR.)STEIN
Cryptochilum sp.
Cyclidium glaucoma O.F.MÜLLER
Cyclidium heptatrichum SCHEW.
Cyclidium singulare KAHL
Cyclidium versatile PENARD
Cyrtolophosis elongata (SCHEW.)
Enchelyodon armatus KAHL
Enchelys farcimen MÜLLER-EHR.
Euplotes affinis DUJARDIN
Glaucoma microstoma SCHEW.

Glaucoma scintillans EHRENBERG
Holophrya atra ŠVEC
Lacrymaria pupula O.F.MÜLLER
Litonotus carinatus STOKES
Loxophyllum niemeccense (STEIN)
Mesodinium acarus STEIN
Mesodinium cinctum CALKINS
Mesodinium rubrum (LEEGAARD)
Microthorax pusillus ENGELMANN
Microthorax sulcatus ENGELMANN
Opisthotricha similis ENGELMANN
Paramecium caudatum EHRENBERG
Philasterides armata KAHL
Pleuronema crassum DUJARDIN
Pleuronema setigerum CALKINS
Pseudoglaucoma musoorum KAHL
Spathidium sp.
Spirostomum ambiguum MÜLLER-EHR.
Spirostomum intermedium KAHL
Trochilia minuta ROUX
Urotricha agilis STOKES
Urotricha farcta CLAP.et LACHM.
Urozona bütschlii SCHEWIAKOFF

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PROTISTS–BIOMONITORS OF MARINE ENVIRONMENT

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An ever increasing attention has been devoted to the study of protists, however in the Baltic Sea, being one of the most investigated parts of the World ocean concerning zoo- and phytoplankton, the study of this group has only been fragmentary until recently (Boikova 1981, 1984; Melvasalo 1981). Lately the Baltic Sea as well as other marine environments have been polluted by toxic and mutagenic substances. But only a few works are available concerning the effect of toxicants on protists, particularly marine ones (Patin 1979; Curds 1982).

To present a scientific approach for developing qualitative criteria of monitoring marine environment, investigations on the ecology of Baltic protists has been carried out to obtain clear picture concerning the effect of heavy metals on ciliates in laboratory and in situ experiments.

ECOLOGICAL INVESTIGATIONS OF PROTISTS (CILIATA, FLAGELLATA) IN THE BALTIC SEA

From 1975 to 1976 ecological and faunistic investigations of benthic ciliates were carried out in four different littoral biotopes (Boikova 1984). Out of the 56 ciliate species, 17 were dominant, the others were subdominant and insignificant species. The annual dynamics showed great fluctuations in ciliate numbers with the maximum development in July and August, its density reaching 1 million 295 thous. ind x m^{-2}. Communities of benthos ciliates are well developed also in winter. Therefore during

abundant development the given group of organisms appears to be an important component of benthos.

Ecologically, faunistic investigations of plankton ciliates in the southern part of the Gulf of Riga were carried out to gain an understanding of the development of ciliates mainly near the river estuaries in the vegetation period (Boikova 1984). Hydrochemical characters of this area play an important role in the formation of the qualitative composition of the fauna; the organisms are represented by 36 species of marine, brackish and fresh-water forms. Maximum development of plankton ciliates was marked in May. At that time the density of ciliates in estuarine areas fluctuated from 1 million 520 thous. ind x m^3 to 4 million 260 thous. ind x m^3.

Ecosystem investigations of two protist groups, ciliates and flagellates (cell size 1-20 µm), were carried out from 1978 to 1984 in different seasons (Boikova 1981,1983,1984). Material was obtained at 96 stations situated in the coastal and open-sea areas, at international monitoring stations. The temporaral, and spatial distribution of ciliates and flagellates is highly unequal either horizontally or vertically. Though the basic mass is accumulated in the layer above the thermocline, the distribution is patchy. Species composition and density of ciliates also vary according to salinity. A higher density is observed in areas where brackish and freshwaters blend. Protists are susceptible not only to temperature and salinity, but to the concentration of biogenous elements as well, particularly the autotrophic form Mesodinium rubrum which at high density actively utilizes the biogenous elements. In separate periods of the season autotrophic flagellates can be an essential part of the producers in the Baltic, that had earlier not been taken into consideration, and the replacement of autotrophic flagellates by heterotrophic ones is closely related to changes in the environment. A rather high biomass of nanoplankton is observed that is typical of the shelf zones of the World ocean.

INVESTIGATIONS ON THE EFFECT OF HEAVY METALS ON BRACKISH WATER CILIATES

Along with finding the indicators for environment assessment of the development of biologic monitoring, a great importance is attached to the "dose-response" relationship revealed by laboratory experiments. Mass and clone cultures of three dominant ciliate species, Paramecium putrinum, Euplotes harpa and Stylonychia mytilus, were selected from natural coastal populations and adapted to laboratory conditions. The following test-criteria were used for assessing the effect of Pb and Zn on the functional activity of ciliates: survival, resistance, division rate, phagocytosis, conjugation, cytochemical reactions (Boikova 1981, 1982, 1983).

Survival rate of mass cultures, resistance of clone cultures of ciliates, division rate

On the basis of short-term experiments (24-120 h), lethal and sublethal concentrations of Pb and Zn were determined as well as the specific response of separate ciliate species. According to a long-term experiment (up to 5 months), it was found that mass cultures of three ciliate species have a normal survival rate at 1.0 and 0.1 mg/l Pb and Zn and 10.0 mg/l Zn levels. During these experiments in the presence of both Pb and Zn at concentrations of 50.0, 10.0 and 1.0 mg/l specimens appeared (no more than 5-10%) being morphologically different from the normal. By Feulgen's reaction it was found that in the cells, division of macronuclei occurring by cytokinesis was completely destroyed.

To study the structure of ciliate populations under a toxic effect, clones were isolated and the resistance of separate clones to the effect of metals at various temperatures (+18 and +4 ^{o}C) was investigated. The resistance was different - with partial or complete death in one clone - and in other clones ciliate reproduction was observed. Heterogeneity of clone cultures was preserved also at low temperature, though with a lower toxicity. Results of agamic reproduction rate study showed that clones with different toxic resistance differed in their reproduction rate as well. Thus, intrapopulation variability

of ciliates in the presence of heavy metals is inherited and can be explained by the physiological polymorphism of protists that may be considered to be the adaption mechanism of the population to toxicity. This should be taken into consideration in eco-toxicological experiments with organisms of mitotic reproduction (protists, unicellular algae).

Phagocytosis

Intensity of phagocytosis depending on environmental factors appears to be one of the criteria for assessing the physiological state of cell-organisms. This criterion is useful also for estimating the effect of various pollutants on protists. Phagocytosis was studied in ciliates of the clone E. harpa, being of a medium resistance, in experiments with added metals for 15 days At Pb and Zn concentrations of 0.1 and 0.05 mg/l, there was no statistically reliable difference in phagocytosis intensity compared to the control. Higher levels of Pb and Zn, up to 0.1 mg/l have a stimulating effect on the rate of phagocytosis. At a metal level of 10.0 mg/l, the formation of food vacuoles falls drastically or stops completely.

Conjugation

Recently, more and more attention is being paid to genetic monitoring in the system of biologic monitoring. Therefore, conjugation is to be taken as a test criterion. In preliminary experiments 30 complementary mating types of ciliates Euplotes harpa and Stylonychia mytilus were selected. Series of experiments were carried out for studying ciliate ability for conjugation during 5 and 35 days in the presence of Pb and Zn concentrations of 50.0, 10.0, 1.0, 0.1, 0.01 mg/l. Under a toxic impact the exchange of genetic information among the complementary clones is disturbed: with the increase of metal level, the amount of complementary ciliate pairs decreases as compared to the control and the conjugation period increases. Conjugation is more inhibited by lead than by zinc which is proved by the cytochemical reaction of hallocyanine to DNA and RNA cells.

The first results proved to be promising for the use of ciliates as model organisms in studying the genetic consequences of pollution. Laboratory investigations showed that the unique biological organization of protists allowing a wide spectrum of test-criteria, determines, to a certain extent, their advantage over the other test organisms of marine environment.

IMPACT OF HEAVY METALS ON CILIATES IN ISOLATED ECOSYSTEMS IN SITU

Model experiments in controlled ecosystems in situ take a special place in the system of biomonitoring representing plankton community response to stress conditions in nature.

Model experiments in situ were carried out in June, July, 1981, 3 m deep in the Gulf of Riga in balloon-shaped isolated plastic bags of 1.3 m^3 of marine water, to test the effect of Pb and Zn by a single addition of metals to the natural plankton communities (bacterioplankton, phytoplankton, zooplankton, protists). The environmental parameters (temperature, salinity, pH, O_2, biogenous elements, metal level) were controlled simultaneously. Species composition and density of organisms were used as criteria for estimating the changes in natural ciliate communities by determining the index of similarity in species composition and the index of dominance (Boikova et al. 1983).

Change in the structure of ciliate community according to the index of similarity

Altogether 30 ciliate species were established during the experiments. In the experimental series of Pb and Zn (Table 1), the control (S_0) was distinguished by high similarity with the sea from the 1st to the 8th day. Addition of metals at various concentrations considerably affected the species composition of ciliate communities on the 4th day. On the 8th day, at some concentrations a partial recovery of ciliate species composition occurs compared to the control, however, further on with a decrease in the similarity index both between the control ecosystem and the sea, and the control and the plastic bags with metals.

Table 1. Change in similarity index (S) of the ciliate species composition

S	Pb/Zn mg/l	Day			
		1	4	8	14
S_o		1.0	0.7	1.0	0.5
S	0.1/0	1.0	0.8	0.8	0.7
S	1.0/0	0.8	1.0	0.8	0.5
S	0.1/0.1	0.8	0.8	0.7	0.6
S	0.1/1.0	1.0	-	0.7	0.6
S	1.0/0.1	1.0	-	0.7	0.6
S	1.0/1.0	0.8	0.5	-	0.3
S	0/0.1	0.7	0.5	1.0	0.4
S	0/1.0	0.7	-	0.7	0.6

Change in the structure of ciliate community according to the dominance index

The dominant ciliate species were determined by the dominance index (Id) that characterizes the role of species in the ecosystem in relation to their occurrence and density. According to the biosamples, the dominant group consists of ciliates Strombidium sp. (Fig. 1). Another picture was observed in the control bag and in the 8 plastic bags with added metals. According to the data of the experimental series, the amount of ciliates Strombidium sp. greatly decreases, on the average, from the 1st to the 4th day compared to the control. Ciliate community showed a distinct response during the first hours of the experiment by lowering their density that was not observed in any other plankton group. Further on in all the isolated volumes with added metals an increase in density of the ciliates Strombidium sp. occurred by 1-2 orders of magnitude till the 14th day, and simultaneously ciliates of the Euplotes sp. were dominant. A similar replacement of the leading species occurred also in the control. Therefore, if the reaction of ciliate community from the 1st to the 14th day can be considered to be a response to

toxicity, later the toxic effect is concealed by the "effect of the bag".

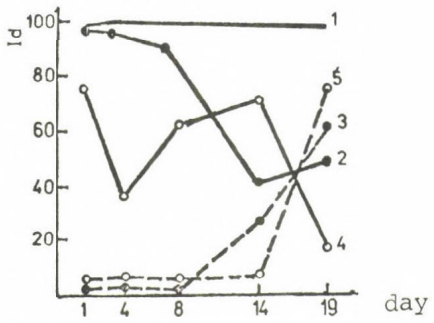

Fig. 1. Development of dominant species of ciliates in an isolated ecosystem and sea: 1 - the sea (Strombidium sp.), 2 - control (Strombidium sp.), 3 - control (Euplotes sp.), 4 - exp. + Pb/Zn (Strombidium sp.) 5 - exp. + Pb/Zn (Euplotes sp.).

The obtained results showed that the natural protist populations were highly susceptible and resistant to the effect of plastics or metals, thus necessitating a rapid control under marine conditions.

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LITONOTUS-EUPLOTES, AN INTERESTING MODEL OF PREDATOR-PREY INTERACTION

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The genus Litonotus is quite well known for its predatory capabilities; Dragesco (1962) was the first to describe the main morphological traits related with its behaviour and compared it with that of other predators.

In our laboratory, a more specific and integrated study of almost the same system (our Litonotus belongs to a marine species, likely L. lamella) was planned and carried on, by comparing four different and complementary aspects of the biology of this predator: A) its ethology, qualitatively and quantitatively described by means of its ethogram; B) prey specificity, if any; C) cell morphological changes caused on the prey by the predator activity; D) ultrastructural analysis of the predator toxicysts.

A) Eibl-Eibesfeldt (1970) gave a definition of ethogram (the precise catalogue of all the possible behavioural patterns of a species) useful also for the Protozoa; the first attempt at applying such a conceptual tool to a ciliate (Oxytricha bifaria) was made by Ricci (1981). Litonotus has been recently studied and its ethogram drawn by Giuntoli et al. (1984). By and large it has been shown that this species behaves in much the same way as the other ciliates so far studied, at least in its motile patterns. The most relevant peculiarity of the behaviour of our Litonotus is the complex of specific reactions shown during the predatory activity. As soon as the proper prey is contacted, a certain number of toxicysts is discharged, then Litonotus creeps backwards for as long as 500-1500 µm. Afterwards, it moves about as if searching for the prey, exploring

the substrate by a series of slowly performed jerks, which eventually leads it to contact and ingest the prey in about 80% of the cases.

B) The problem of prey specificity was tackled by offering to the predator as potential preys different species of ciliates, namely: Diophrys scutum; Condylostoma arenarium; Frontonia sp.; Aspidisca pulcherrima; Euplotes crassus and Euplotes minuta. Quite a few individuals per species were mixed together or many individuals of a single species were offered to hungry Litonotus. In both cases only individuals of the two Euplotes species were predated. No specific searching activity by the predator could be demonstrated; the toxicyst discharge immediately followed the casual contact between Litonotus and Euplotes. The contact with the other species did not elicit any toxicyst discharge by the predator. How can such a very specific and instantaneous recognition be obtained? Plasma membrane should be considered as the recognition target in the predator-prey interaction. A first series of experiments was carried out to obtain non-specific membrane perturbations by treating Litonotus with enzymes like protease, alpha amylase, diastase, hyaluronidase and lectins like PHA and Con-A. All the above-mentioned substances were used from 0.05 up to 2% (w/v). A second round of experiments is now in progress testing the same substances on the prey. The results obtained show that the cell recognition occurring when the predator bumps against the prey is mediated by mechanisms not affected by any of our experimental treatments. The mechanisms at work in the Litonotus-Euplotes interaction appear to differ from those mediating the prey-predator interactions in Dileptus (Esteve, 1981), which are affected by Con-A and protease given to the predator.

C) Ciliary morphological modifications caused by the predator attack on the prey have been analyzed (Verni, 1985). First of all, the AZM and the cirri of Euplotes lose their typically compact morphology, with the cilia becoming disarranged in single units. Besides, almost all of the single cilia of the AZM and cirri undergo a modification at their terminal portion. At this level the axoneme becomes folded, and the ciliary membrane enlarges greatly into a rounded vesicle (paddle-like

cilia) with a diameter of about 1 μm. It is worth mentioning that only the locomotory cilia of the prey (AZM and cirri) are affected by this phenomenon, while the dorsal bristles of poisoned Euplotes do not change their form. Bristles are usually assumed to have sensory functions; in particular, Gortz (1982) suggests that the bristle complex of hypotrichs has a role in the mechano-sensory transduction.

In Turbellaria, under special conditions, the formation of paddle-like cilia concerns only the sensory cilia (Ehlers and Ehlers, 1978) while the locomotory cilia maintain their normal tip. On the contrary, the results reported above indicate that the content of the toxicysts of Litonotus acts on the motile ciliature whereby the Euplotes prey becomes paralyzed forever. The paralyzed prey is doomed to die even if it escapes the search of the predator. On the other hand, the predator is able to suck into its cytoplasm only paralyzed prey.

D) Ultrastructural and cytochemical analyses of the two types of toxicysts of Litonotus are now in progress. The ultrastructure of these extrusomes follows the general scheme known in other gymnostome predators: they consist of fully developed structures inside a capsule. However, some differences can be observed even between the two species of Litonotus examined till now, namely L. quadrinucleatus (Bohatier and Njiné, 1973) and the marine Litonotus under study. In the latter an external granular layer, lacking in the former species, surrounds the capsule of one type of toxicysts. The cytochemical analysis performed by means of enzymatic procedures shows that this layer is partially affected by RNAse and completely by protease. This is indicative of the presence of ribosomes in this layer. At this level, acid phosphatase activity has also been found. In the other type of toxicysts this same enzyme is present in the internal matrix. It is possible that the toxicysts with the external layer may represent a maturation stage of the true toxicyst, the one which is ejected. The acid phosphatase could be synthesized in the granular layer and then it enters the internal tubule for its extrusion. However, the two types of toxicyst could actually be different extrusomes with distinct function as the pexicysts and toxicyst of Didinium (Wessemberg

and Antipa, 1970). In all four parts touched upon in this short presentation, the questions still open are more numerous than those answered. We hope to find an answer at least to some of them.

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STRUCTURAL INVESTIGATIONS OF PERIPHYTIC PROTOZOAN COMMUNITIES IN THREE LAYERS OF THE DANUBE RIVER. III. ANALYSIS OF THE SAPROBITY RELATIONS (DANUBIALIA HUNGARICA CVII)

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Protozoans are without doubt of great importance from the points of view of both the natural living systems and human activity. Their significance regarding the latter cannot be restricted only to the medical and/or veterinary aspects, as their role in the process of self-purification, or their use as indicator organisms is just as important.

Investigations of the planktonic protozoa communities have been carried out at our institute for almost two decades (Berecky, 1969, 1971, 1973, 1975-1979, etc.) Several years ago we have started with the study of the periphytic protozoan communities, too.

The aims of our periphyton studies were to determine the species composition, abundance relations of the periphytic protozoan community and to follow the temporal changes of the former phenomena, that is the course and rate of colonization.

It is commonly accepted that in lakes, especially in deep ones, there is a stratification. Our investigations from the beginning were directed to the question of whether vertical stratification exists in running waters or not. Therefore among the abiotic factors examined, particular attention was devoted to depth.

Investigations were carried out at Göd, at the river km mark 1669, in different seasons of different (and characteristic) water flow regimes (Fig. 1).

Microscopic glass slides were applied as artificial substrata, exposed in three depths: immediately under the water surface; in the middle of the water column and near the bottom.

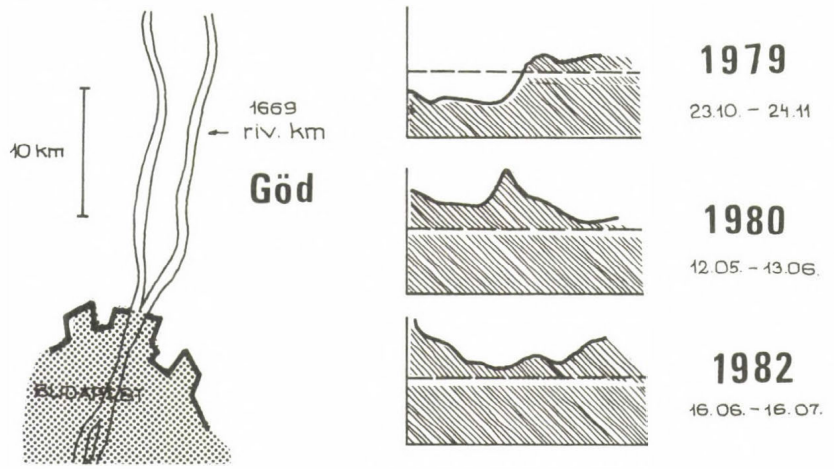

Fig. 1. Location of the sampling site and the water discharge curves of the experimental periods. Broken line indicates the average discharge of the Danube.

Fifty per cent of the slides were coated with ovalbumin in order to investigate the effect of a preliminary nutrient resource on the colonization process. The other half of the slides was left untreated.

Samples were taken on the 2nd, 4th, 8th, 16th and 32nd day after the exposure. Simultaneously, samples were taken from the plankton. The relative abundances of the different saprobic groups were determined: that is, that of oligo-(o), oligo-beta-(o-b), beta-(b), beta-alpha-(b-a), alpha-(a), alpha-poly-(a-p) and polysaprobic-(p) organisms. Values of the former three and latter four groups were summed, and the calculations were made based on these two sums.

A detailed description of the methods and sampling process is to be found in Part I (Bereczky et al., 1983).

Although this paper is devoted to the saprobity relations, we cannot avoid outlining briefly the results of the other former investigations (see also Bereczky et al., 1983, Nosek and Bereczky, 1983, Bereczky, 1985).

From the number of species, total number of individuals and diversity, using multivariate analysis of variance, in certain cases a stratification could be detected. That is, the layers

near the surface and those near the bottom have always been separated when this stratification occurred.

In the case of the physico-chemical parameters and the plankton the separation of these two layers could be observed only in the fall of 1979 (at a constant and low water flow regime). With the periphyton, stratification occurred every year. The course of colonization was different at the different depths and on the different slides. The number of species and individuals was higher in the layer near the bottom and on the treated slides than in the surface and on the untreated slides. The rate of colonization was also higher in the layer near the bottom and on the treated slide.

The advantage given by the preliminary nutrient source was effective only in the early period of colonization, and manifested in the greater values of the parameters or in the damping of the oscillations of the parameter values.

Summarizing the results on the basis of the number of species, absolute abundance and diversity, it can be said that stratification could occur in running waters, too, and the layer near the bottom seems to be a more favourable environment for the protozoans than the one near the surface.

Regarding the saprobity relations, in the case of the plankton no stratification could be detected in any of the years. The diagrams show the familiar beta-mesosaprobic character of the Danube water (Fig. 2).

The lack of stratification in 1979 seems to be contrary to the results gained from the other parameters. In 1979 the bottom layer had a greater absolute abundance than the upper one. But thinking over the facts, there is no contradiction, as the relative abundance may be the same at different absolute abundances.

Within a single experimental period there were no remarkable differences among the samples. Comparing the separate years, a tendency toward a better water quality may be observed. The proportion of the polysaprobic group decreases while that of the oligo-beta and beta-mesosaprobic groups increases.

The picture in the case of the periphyton is quite different. Table 1 shows the results on the analysis of variance.

Table 1. Result of the analysis of variance. Level of significance, $p<5\%$

Source of variance	Σ (o,o-b,b) 1979	1980	1982	Σ (b-a,a,a-p,p) 1979	1980	1982
Untreated slides						
Duration	x	x	x	x	x	x
Depth	ns	x	x	ns	x	ns
Interaction	x	x	x	ns	ns	ns
Treated slides						
Duration	x	x	ns	x	x	x
Depth	x	x	ns	x	x	ns
Interaction	x	x	ns	x	x	x

The duration of the exposure is significant (x) in almost every case. There were great differences among the periphyton samples of different ages within the same experimental period. Due to lack of space, in Fig. 3 only a few diagrams are presented as examples.

The results of the second and fourth days cannot be accepted as characteristic of the periphyton because of the low number of species and individuals. This period is the early stage of the development. Former results have indicated that the periphyton reaches its maximal development between the 8th and 16th days. Therefore the saprobic relations indicated by these samples reflect the real situations within the periphyton.

When stratification could be demonstrated, the surface and the bottom layers had been separated, as with the earlier results. In 1979 the layer near the bottom and in 1980 the surface layer showed better water quality (Table 2, Fig. 4).

Table 2. The difference of means on the basis of MANOVA (s - surface-near; b - bottom-near layer)

	Σ(o, o-b, b) 1979	1980	1982	Σ(b-a, a-p, p) 1979	1980	1982
Untreated slides	-	s>b	s>b	-	s<b	-
Treated slides	s<b	s>b	-	s>b	s<b	-

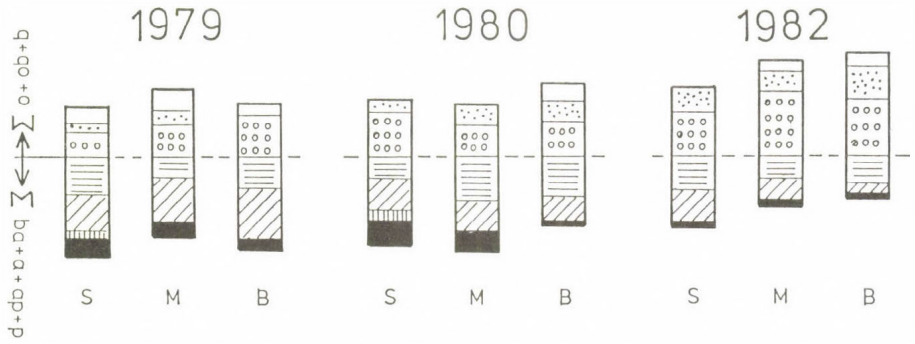

Fig. 2. Relative abundances of the different groups in the plankton samples of the 8th day in the separate years.

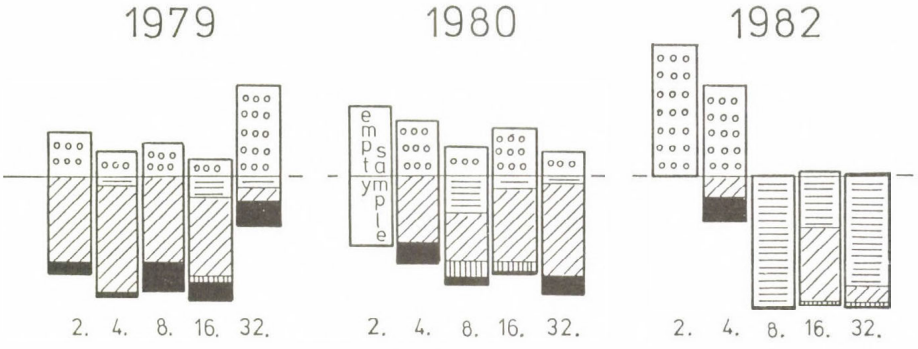

Fig. 3. Changes in the relative abundance of the different groups on the untreated slides during the development of the periphyton in the surface near layer.

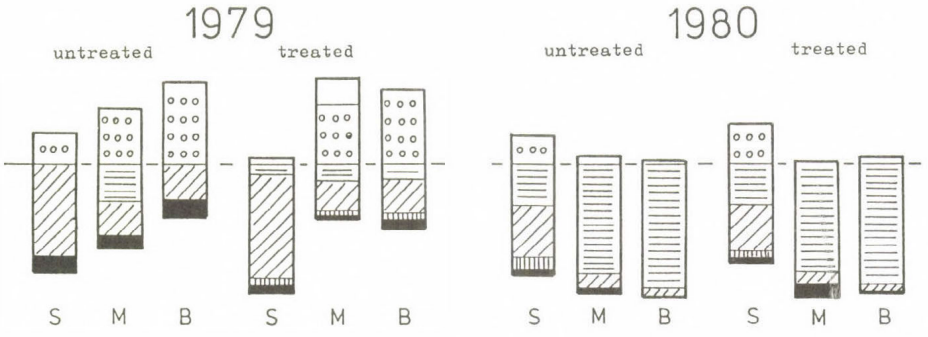

Fig. 4. Relative abundances in the periphyton samples of the 8th day on both types of slides (S = surface near, M = middle, B = bottom near layer).

The significance of the interaction refers to the fact that the two factors, depth and duration of the exposure, are not independent of each other. That is, the difference between the layers, the stratification, depends on the development of the periphyton and does not occur in the early stage.

Regarding the separate seasons, there are also great differences among them.

Comparing the diagrams of the plankton and the periphyton, the smallest difference can be established in 1979, at the constant, low water flow regime. In other seasons, with greater water discharge, this difference increases. It can also be established that the periphyton always indicates a little worse water quality (cf. the corresponding diagrams of Figs 2, 3 and 4).

Summarizing these facts, the picture we have got about the saprobity relations of the periphyton is much more diverse than the picture of the plankton.

The protozoan periphyton has its own life history with a series of different stages along which species with different feeding habits follow each other. We may say, the protozoa periphyton undergoes its own trophic succession series. The identity of the first invaders, that is those which will occur first on the bare substrata, depends heavily on chance. The role of chance factors remains during the development, but with decreasing importance as the self-governing processes of the periphyton become more and more stronger. That is, the temporal changes of the periphyton are more independent of the surrounding water than in the case of the plankton.

Moreover the organic detritus accumulates on the substrata. The amount of the organic matter to be decomposed increases with time in the periphyton, partly owing to its own development. This phenomenon does not occur in the pelagic habitat, detritus is sinking down and accumulates over the bottom. Therefore the saprobic conditions indicated by the protozoa periphyton are much more valid for the periphyton itself than for the water body in which the periphyton has been developed. In other words, for the qualification of natural and waste

waters the planktonic protozoa communities seem to be more appropriate.

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INFUSORIA-SUBSTRATE RELATIONSHIPS IN THE SMALL STREAMS OF LATVIA

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The structure of biocenoses of protozoan benthos was investigated in the small streams of Latvia not being under the anthropogenic impact. To compare the biotopes according to protozoan benthos the index of the total species diversity H (Odum, 1975) was used as well as indices of dominance Id and frequency V (Schwerdtfeger, 1975).

Differences in water stream biotopes are determined by the relief (stream velocity, type of sediments) and the situation in the river bed. The streams are constantly added to by substances from land ecosystems therefore the composition of sediments is closely related to it (streams running through forests, agricultural areas, swampy meadows, areas where land reclamation is being carried out, such as deepening of beds, regulation of streams). Due to similarity of communities in physico-graphically similar biotopes, while certain species in a biotope occupy identical niches (Illies, 1961), in all the investigated streams 4 different habitats were distinguished: sand, gravel, silt, peaty silt. Microdistribution of protozoan benthos, of course, is determined by a complex of factors, such as temperature, sediment composition, depth of the water body, chemical indices, and not by one of them separately (Bartsch, Hartwig, 1984; Madoni, 1983, 1984; Schmitz, 1983). However, when investigating the small streams we considered only the type of sediment because of three reasons: firstly, in the contact layer "water-bottom" the effect of stream current is indirect (Ambühl, 1962), secondly, as the

investigations were carried out during the vegetation period the thermal conditions were similar in them, and thirdly, the water depth in the sampling sites did not exceed 1.5 m.

On river bed shelves and riffles the basic sediments was sand in all the investigated streams. Usually sand bottoms have organic debris admixture.

Five infusoria species (Coleps hirtus Nitzsch, Dileptus anser (O.F.M.), Frontonia leucas Ehrb., Pleuronema coronatum Kent, Uroleptus rattulus Stein) out of 49 stated on sandy bottoms appear to be constant, 4 of them are eurytopic. The dominating group (10 species) consists mainly of ubiquists (Coleps hirtus, Dileptus anser, Euplotes patella (O.F.M.), Frontonia leucas, Lacrymaria pupula O.F.M., Paruroleptus piscis (Kow.), Pleuronema coronatum, Urocentrum turbo (O.F.M.), Urostyla grandis Ehrb., Uroleptus rattulus). Seven infusoria species (Chaenea limicola Laut., Climacostomum virens (Ehrb.), Euplotes affinis Dujardin, Paruroleptus caudatus Stokes, Pseudoprorodon farctus Clap.e.L., Prorodon ovum (Ehrb.), Saprophilus putrinus Kahl) are bound only to sandy biotopes. Detailed investigations on the structure of Infusoria communities in sandy sediments showed that everywhere alongside with oligo- and mesosaprobic forms (Dileptus anser, Frontonia acuminata Ehrb., Homalozoon vermiculare Stokes, Pleuronema coronatum) polysaprobic ones were observed as Caenomorpha sapropelica Kahl, Metopus contortus Quenn., M.striatus McMurrich, Paramecium caudatum Ehrb., Saprodinium dentatum Laut., only their density differed (on the average 200 thous.ind./m^2 and 40 thous.ind./m^2, respectively). Obviously, a primary importance in forming the structure of such communities belongs to the velocity of current. Due to that the contact layer water-bottom and the sand surface is always abundantly supplied with oxygen solubilized in water and the products of metabolism are carried away, that means the organisms of clean waters are under optimum conditions. On the other hand, the current mobilizes uncirculating biogenous substances from the bottom, sorts various fractions of the sediment surface, brings them into contact layer and

makes them available for the first member of the trophic chain - microorganisms, and further on for infusoria. It can be traced most clearly at the investigation of infusoria distribution within a microbiotope. Thus, small deepenings (1-2 cm) accumulate silt, half destructed plant remnants, etc. In this temporary microbiotope saprophytic forms develop rapidly due to short reproduction period. Indicator of the total species diversity (H) on sand has a rather wide range of fluctuation (0.6-2.6), on the average being 1.5. A positive correlation between the total number of species on one hand and density on the other hand can be detected on sand. It can be obviously explained by the fact that at higher species number systematically close species of one biotope have different ecological niches, and that lowers the competition among them.

In order to characterize the food supplement for Infusoria the ratio of the total amount of microorganisms and the total amount of infusoria was used (A_m/A_i). On sand this indicator was 1.32, that means infusoria are completely supplemented with food (Table). It should be taken into consideration that Infusoria are able to select not only among the food objects of different groups (bacteria, phytoplankton, detritus, etc.) but also within one group. For example, infusoria of one water body, systematically even very close, is a food object of various microorganisms (Taylor, Berger, 1967). On sand we have ooserved a positive correlation between the total amount of microorganisms and density of Infusoria (r=0.6). The given correlation, of course, does not reflect completely the interrelationships between bacteria and Infusoria. The correlation was calculated using the total number of microorganisms because of the fact that food vacuoles of Infusoria are filled with detritus and algae, but there are also microorganisms adsorbed on them. On sandy bottom we failed to observe a correlation between the total density of protozoan benthos and the amount of organic stuff. The same can be said about the total number of bacteriobenthos (Veilande, Liepa, 1985). Obviously, for their development the qualitative

composition of organic stuff accumulating on the sediment is also important.

More seldom the river bed is formed of gravel. On gravel 49 infusoria species have been found, 7 of them constant (Coleps hirtus, Euplotes patella, Frontonia leucas, F.acuminata, Loxodes magnus Stokes, Paruroleptus piscis, Spirostomum minus Roux). In the given community 13 species of infusoria are dominating (Coleps hirtus, Euplotas patella, Frontonia leucas, F.acuminata, Loxodes magnus Stokes, Loxocephalus plagius (Stokes), Lembadion magnum (Stokes), Paruroleptus piscis, Pleuronema coronatum, Strombidium viride Stein, Urocentrum turbo (O.F.M.), Uroleptus rattulus, Urostyla grandis). According to lists mentioned before, everywhere the dominant species are of eurytopic form. The stenotopic group comprises 9 species: Caenomorpha medusula Perty, Cinetochilum margaritaceum Perty, Condylostoma vorticella (Ehrb.), Cyclidium citrullus Cohn, Dileptus cygnus Clap.e.L., Glaucoma scintillans Ehrb., Paramecium bursaria Ehrb., Pseudoprorodon foliosus Foissner. On gravel the richest species diversity is observed - 1.8 on the average, and it should be noted that this indicator exceeds 1.5 in all the investigated gravel biotopes.

In gravel biotopes the species diversity of Infusoria has no positive correlation with their total amount, but according to the negative coefficient of correlation the increase of the total number depends on higher reproduction of separate infusoria species. Quite possible that on gravel increased taxonomic diversity of Infusoria can be explained by numerous "micro water bodies" forming among the gravel grains where hydrochemical regime and food availability may differ forming microcenoses of Infusoria with high species diversity. However, in a limited temporary space competition increases concerning the food objects and habitats, and generally an increase of the total number of infusoria has not been observed in such biotopes. Probably it may be due to the fact that gravel is a less favourable environment for bacteriobenthos, as 1 g of wet sediment contains 104.0 to 246.0 million microbic cells, that on the average, is 1.6 times less than, e.g., on sand (Veilande, Liepa, 1985).

In some reaches of the small streams there is black silt on the river bed. On silty bottoms 39 infusoria species were found. Five of them were constant (Frontonia leucas, Lembadion magnum, Spirostomum minus, Strombidium viride, Uroleptus rattulus). The dominating species were seven (Coleps hirtus, C.hirtus var.lacustris Faure-Fremiet, Frontonia leucas, Lembadion magnum, Spirostomum minus, S.teres, Strombidium viride), but six species were bound only to black silt biotopes (Blepharisma undulans Stein, Chilodonella cucullulus (O.F.M.), Condylostoma tardum Penard, Glaucoma myriophylli Penard, Plagiopyla nasuta Stein, Zoothamnium arbuscula Ehrb.). The index of species diversity on all the investigated biotopes was always above 1, making 1.1 on the average (Table); the average number of separate species was 580 thous.ind./m^2. In reaches where current velocity falls to minimum (0.2 m/sec), and in separate cases there is no current at all, i.e. the environment may be considered lentic, the ecological conditions are more monotonous, due to abundance of food for bacteriophage Infusoria (e.g., spatial density of microorganisms is the highest reaching an average of 407.0 million cells per 1 g of wet sediment), and due to the lack of food competition a smaller number of species reach high development. Comparison between the number of species and the total density of infusoria reveals a positive correlation.

In areas where rivers run through swampy meadows the river bed is formed of peaty silt. The total amount of infusoria species in peaty silt is 26. Four of them are constant (Coleps hirtus, Lacrymaria pupula, Loxodes magnus, Spirostomum teres) and 6 dominant species (Coleps hirtus, C.hirtus var.lacustris, Frontonia leucas, Lacrymaria pupula, Loxodes magnus, Spirostomum teres). Four species (Holosticha grisea Kahl, Prorodon viridis (Ehrb.), Stentor mülleri (Bory St. Vincent), Urosoma cienkowskii Kow.) can be considered as stenotopic. Index of species diversity on these biotopes is the lowest (Table), though the average number of separate species is the highest, and there is a positive correlation

between these indices. Although peat appears to be an environment rich in organic stuff, its chemical composition prevailed by humic substances determines a weak development of bacteriobenthos - 1 g of wet peaty silt contains on the average 155.5 million cells (Veilande, Liepa, 1985). On the other hand, bacteria are more fed on by Infusoria here, their total density on peat being the highest while the environment limits development of organisms feeding on infusoria.

The average data of Infusoria in small streams

Biotopes	Index of species diversity (H)	Amount of Infusoria thous.ind./m^2	A_m/A_i
Sand	1.5	191.7	1.32
Gravel	1.8	273.3	0.67
Silt	1.1	579.2	0.70
Peaty silt	0.9	646.8	0.24

Altogether 177 infusoria species have been observed in the investigated streams, and 12 of them are eurotopic. These species are the basic components of all four sediments. Ecologically they are elastic being resistant to the changes in hydrochemical conditions and the character of river bed sediments. They are part and parcel of the protozoan benthos community in loose and solid substrate of the surface layer.

Investigations of various biotopes in relatively undisturbed river parts showed a positive correlation between the number of infusoria species and their total amount. This general regularity has not been observed in gravel sediments. On hard sediments (sand, gravel) the species diversity is higher than on loose ones where the community structure is simpler, but the quantitative development of Infusoria is higher. The stability of habitats is the basic factor regulating the species diversity in the cenosis (Alimov et al., 1982). It is higher in older communities. Probably on the border line between lentic and lotic environment where the current velocity falls to 0.2 m/sec a continuous sedimentation of organic stuff, either autochthonous or allochthonous

one, occurs on the bottom surface, i.e. there is no equilibrium of processes.

On sand and gravel the trophic bounds of unicellulars were more branched therefore with the changes in environment inner reorganization may occur until certain time without significant changes in the cenosis in general.

On loose sediments the decrease in species diversity is bound to the decrease in ecological niches. Therefore irreversible changes may occur with the changes in environmental factors. That is proved also by the increase in eurytopic species number, by the decrease of stenotopic as well as constant and dominating group.

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SEASONAL CHANGES IN CILIATE POPULATION IN VRISHBHAVATHI STREAM, BANGALORE UNIVERSITY CAMPUS, BANGALORE, INDIA

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Protozoans have world wide distribution, and they have excelled in their ability to adapt themselves to widely varying habitats. There are no environmental niches except the air, where protozoa have not learned to live.

Protozoans are known to be heterogeneous, samples collected from visually similar habitats or within a small area may show no tendency to be similar in protozoan fauna.

The ecological adaptation is an important factor in determining the geographical distribution of protozoa. Some of the important ecological factors limiting protozoan distribution are: Temperature, pH, dissolved oxygen, dissolved carbon dioxide, nutrients, and finally the interaction amongst themselves and with other organisms.

The density of population is very important because of its influence on the main biological processes. It has been shown that the density of population has a pronounced influence on the longevity and fertility of the organisms (Pearl 1925).

Vrishabhavathi stream is a tributary to the river Arkavathi, which in turn joins the river Cauvery. Vrishabhavathi stream runs along one side through Jnana Bharathi Campus, Bangalore University. Hundred yards away from the main entrance of Bangalore University, the stream is joined by a sewage outlet. In the present study, field and laboratory investigations were carried out to determine the ciliate population, and their relationship with different ecological parameters. The present study involves six sites in the Vrishabhavathi stream, 3 sites, viz. VR1, VR2 and VR3, within the campus premises. Our

investigation has shown that the population density of a given species is not the same in different parts of its vital zone. The density of population reaches its highest value in definite optimal conditions and decreases in such habitats where the conditions deviate from the optimum.

Many studies have indicated that the abundance and distribution of food organisms are major factors in determining the abundance and distribution of protozoa (Noland 1935, Lackey 1938, Staut 1956). The relationship between bacteria in aquatic ecosystem and the ciliate protozoan has created much interest because of the increasing urgency of understanding the effects of organic enrichment on aquatic system and in secondary productivity and nutrient cycling (Bott 1976, Siebarth 1976). Many investigators have studied the growth rate of ciliate to the density of their bacterial prey (Canale et al. 1973, Berk et al. 1976, Ashby 1976). The organic matter content of the stream varies along their length because of the inflow of organically polluted water from tributaries (Hynes 1960). The rate of growth of protozoa alters with the concentrations and the type of changes in the culture media.

Seasonal variation of protozoan fauna was observed in Vrishabhavathi river which might be due to the above mentioned various changes that take place in the water. Seasonal fluctuations of protozoa were reported by Crozier (1923) in sewage filter.

MATERIALS AND METHODS

The samples from the designated sites were collected in wide mouthed bottles of 500 ml capacity. About 250 ml samples in triplicate were collected from each of the designated sites in wide mouthed bottles. Immediately after reaching the laboratory the samples were analysed and the ciliate populations present at each site were recorded. The density of ciliate population was determined by counting the different species in a known volume of the sample. In most cases the ciliates were classified and identified by taking body measurements (shape,

size, etc.) with the help of calibrated ocular micrometer. Different staining techniques like methyl green, Feulgen and silver impregnation techniques were employed to study the nuclear shape, size and infraciliature of the ciliate.

Water samples were analysed for the following parameters: water temperature, pH, dissolved oxygen, dissolved carbon dioxide, silicate, phosphate, nitrite, nitrate and dissolved organic matter. Water temperature, pH and dissolved carbon dioxide were recorded on the spot and other parameters were analysed in the laboratory.

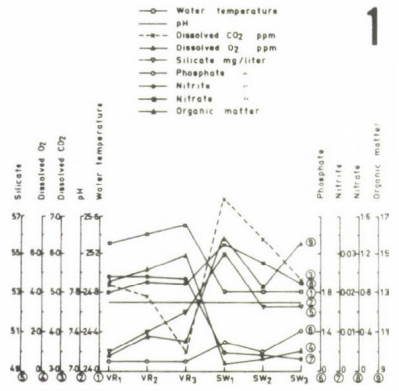
The amount of dissolved carbon dioxide was estimated on the spot by standard titration method of Benton. Dissolved oxygen present in the sample was estimated by Winkler's azide modification method. Total dissolved phosphate, nitrate, nitrite and organic matter silicate present in the sample were determined colorimetrically by Thingran method.

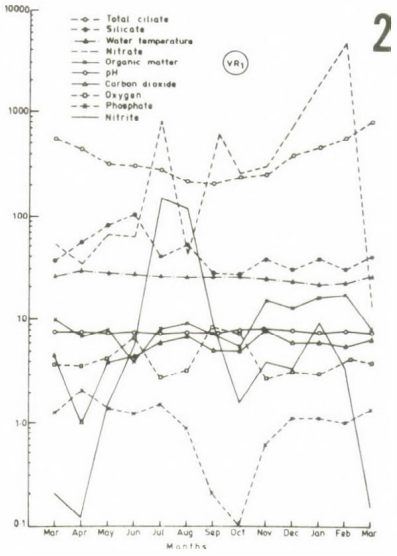
OBSERVATION

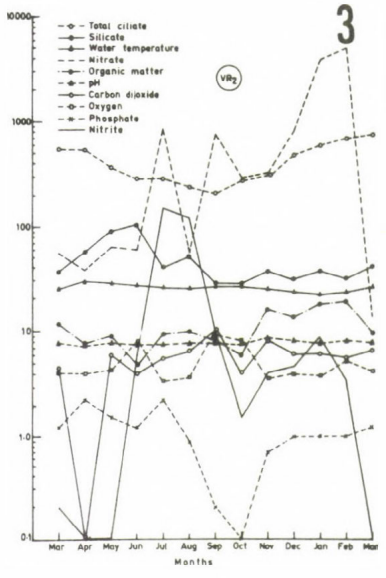
The present investigation of the Vrishabhavathi stream running along one side of Jnana Bharathi Campus, Bangalore University, Bangalore, revealed the distribution of ciliates in relation to their habitats. The density and distribution of ciliates is closely connected with the ecological factors like food, temperature, pH, dissolved oxygen, dissolved carbon dioxide, silicates, phosphate, nitrite, nitrate and organic matter.

Figure 1 presents the ecological conditions of different habitats studied; in Vrishabhavathi stream, namely VR1, VR2 and VR3.

Figures 2, 3 and 4 give the density of ciliates during the different months in sites VR1, VR2 and VR3. In site VR1 densities of Vorticella microstoma and Epistylis plicatilis were at peak during March. Density of V. campanula was high during April. Frontonia leucas was plenty during May. Stentor polymorphus, S. roseli, P. caudatum, L. fasciola, Didinium nasutum were at highest density during June, E. plicatilis in August,







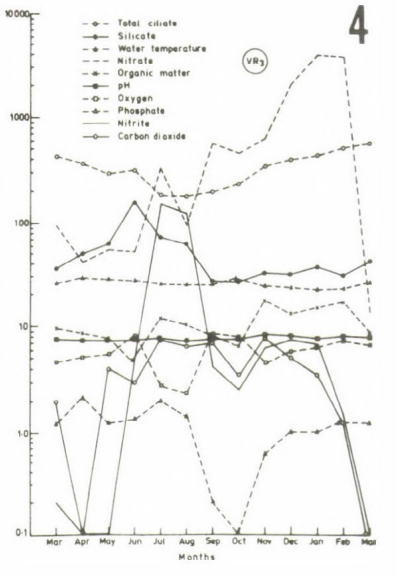


Figure 1. Graphic representation of the average data of ecological factors of six habitats studied (VR1, VR2, VR3, SW1, SW2, SW3).

Figures 2-4. Graphic representation of the ecological factors and the total density of ciliate population during different months in natural habitats in VR1, VR2, VR3.

V. striata, E. woodroffi, E. patella, Oxytricha setigera and P. aurelia during October, Stylonychia putrina, Blepharisma intermedium, Paramecium multimicronucleatum, Zoothamnium adamsi and Spirostomum ambigum during November, V. convallaria during January and Campanella umbrellaria during February. Similar fluctuations were observed in other sites, too.

Table 1 gives the percent density of peritrichs, holotrichs, hypotrichs and spirotrichs during different months of study, in different sites, VR1, VR and VR3. In all the six sites the density of peritrichs was found to be higher than in other groups. In sites VR1, VR2 and VR3 the peritrich density was highest during the months February to April. Whereas holotrichs were high during August and September, spirotrichs were high during June and July, hypotrich peak density was noticed as follows: in VR1 during September, in site VR2 during November and in site VR3 during May.

DISCUSSION

Relative abundance of an organism in a habitat is often used as a guide to indicate its importance in ecological structure of that habitat. Contributions to the ecology of different habitats have been made by Woodruff (1913), Bodine (1921), Peters (1921) and Philips (1922).

Distribution and abundance of protozoa, in particular the peritrichs in natural habitats, depict the ecological condition of that habitat, for example Vorticella microstoma thrives in extremely polluted water, but V. campanula perishes in such environment (Finley 1966), Carchesium and Vorticella can adapt to fluctuation in total alkalinity, dissolved oxygen, ammonia, nitrite, nitrate, phosphate, sodium chloride, light, temperature, pH, etc., whereas Epistylis and Zoothamnium do not adapt to these fluctuations. Cothurnia and Pyxicola thrive well in low dissolved oxygen but do not live in ponds with higher concentration of dissolved oxygen (Finley 1966).

Table 1. Density of ciliates (holotrichs, hypotrichs, spirotrichs and peritrichs during the period of study in sites VR1, VR2 and VR3

Months	VR_1				VR_2				VR_3			
	Peri.	Holo.	Hypo.	Spiro.	Peri.	Holo.	Hypo.	Spiro.	Peri.	Holo.	Hypo.	Spiro.
1982												
March	84.0	9.0	5.0	2.0	83.0	9.0	4.0	4.0	78.0	9.0	4.0	9.0
April	74.0	14.0	7.0	5.0	81.0	9.0	7.0	3.0	79.0	11.0	5.0	5.0
May	56.0	23.0	11.0	10.0	53.0	27.0	10.0	10.0	50.0	21.0	14.0	15.0
June	47.0	25.0	14.0	14.0	47.0	31.0	4.0	18.0	43.0	21.0	13.0	23.0
July	48.0	18.0	20.0	14.0	53.0	25.0	6.0	16.0	45.0	27.0	13.0	15.0
Aug.	42.0	24.0	27.0	7.0	50.0	31.0	8.0	11.0	54.0	25.0	13.0	8.0
Sept.	41.0	25.0	29.0	5.0	50.0	31.0	8.0	11.0	41.0	30.0	11.0	18.0
Oct.	44.0	21.0	26.0	9.0	50.0	21.0	2.0	9.0	54.0	26.0	9.0	11.0
Nov.	62.0	18.0	13.0	7.0	58.0	19.0	14.0	9.0	59.0	21.0	8.0	12.0
Dec.	75.0	13.0	5.0	7.0	74.0	12.0	5.0	9.0	65.0	14.0	7.0	14.0
1983												
Jan.	81.0	9.0	4.0	6.0	80.0	9.0	4.0	7.0	78.0	9.0	4.0	9.0
Feb.	86.0	8.0	3.0	3.0	84.0	8.0	3.0	5.0	77.0	11.0	6.0	6.0
March	86.0	6.0	2.0	6.0	84.0	8.0	4.0	4.0	74.0	11.0	9.0	6.0

Peri. = peritrichs; Holo. = holotrichs; Hypo. = hypotrichs; Spiro. = spirotrichs

Population dynamics of ciliates under a variety of environmental conditions have been investigated by Bick (1964) and Munch (1970).

The different ecological factors also show seasonal variations. Bamforth (1958) recorded the changes in dissolved oxygen, carbon dioxide, light, temperature, pH, nitrite, nitrate, ammonia and phosphate in a small artificial pond. The oxygen curve showed a maximum in winter and spring and a minimum during summer and fall.

The present investigation of different ecological factors in sites VR1, VR2 and VR3 has confirmed the seasonal variations. The temperature was maximum during March, April and May in all the three sites. pH was maximum during November in sites VR1, VR2 and VR3. Dissolved oxygen was maximum during September in sites VR1, VR2 and VR3. The silicate content was found to be maximum during May-June in all the sites. Dissolved carbon dioxide was maximum during November in sites VR1, VR2 and VR3. Phosphate was maximum during April-July in sites VR1, VR2 and VR3. Nitrite was more during July-August in sites VR1, VR2 and VR3. Dissolved organic matter showed maximum during February in sites VR1, VR2 and VR3.

The correlation between the density of population and different ecological parameters varies from species to species and from site to site. Roux (1901) attributed the seasonal occurrence of ciliates to availability of food, absence of enemies, and the increase in their reproductive rate. Bamforth (1958) pointed out the seasonal development of various planktonic phytoflagellates, which constitute preferential food for ciliates. Oxygen tension of the medium influences the growth and distribution of protozoans (Beedle and Nilson 1959, Goulder 1974). Carbon dioxide is a primary adverse factor limiting the occurrence and distribution of ciliates (Stout 1956). In the present investigation seasonal fluctuations of ciliated protozoa correspond with the seasonal changes in ecological parameters.

SUMMARY

Field investigations have shown that the population density of different ciliates is not the same in different parts of the stream. Different ciliates showed seasonal fluctuations in different habitats studied. The peritrichs were at their highest density during March-April, whereas holotrichs during August-September, hypotrichs during September and November, and spirotrichs during June-July in Vrishabhavathi stream.

The vegetation of the stream also showed an influence on the density of the ciliate population especially on the peritrich population. During early summer months, when the floating aquatic plant Eichornia becomes abundant, the peritrich population also reaches its full bloom and as the water plants number decreases so also the peritrich population.

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ECOLOGY OF PELAGIC CILIATES IN SOME FISHPONDS OF THE NORTHERN WALDVIERTEL

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INTRODUCTION

The northwestern Part of Lower Austria is called Waldviertel. A typical feature of this landscape is the high number of ponds used mainly for fish production with carp as the main fish.

The aim of this work was to get some information about the importance of pelagic ciliated protozoa in fishponds, and to look for correlations with chemical and biological data.

Gates 1984, Hunt & Chein 1983 and Pace & Orcutt 1981 investigated planktonic protozoa in recent time, considering their significance to other faunal components and emphasized the importance of protozoa in lake ecosystems.

METHODS

Samples were collected by a 1-Liter Ruttner bottle and fixed with 1.25 % mercury (II) chloride. Seperate water chemistry and crustacean samples were taken concurrently. Previous studies showed that a time interval of approximately ten days and an integrating sampling strategy (ten sampling points positioned along a transect) are absolutely necessary to get representative data. Subsamples were settled in Utermöhl chambers and counted with an inverted microscope at 200 x magnification. Biovolume estimates based on measurements and approximations of shape to standard geometrical configurations. Wet weight biomass values were derived from biovolume, assuming unity, $10^9 \mu^3 = 1$ mg.

RESULTS AND DISCUSSION

Data are available from five ponds. Haslauer Teich and two ponds called Steingrabenteich 1 and 2 were investigated in 1983 and 1984, Ehrendorfer Teich and Winkelauer Teich only in 1984. These ponds have an area between 0.7 and 50 ha, mean depth differs from 1 to 3 m. Pond management, for example fertilazation and feeding, is influencing each of these ponds in different ways.

Referring to ciliate densities, it can clearly be noted that numbers of ciliated protozoa reach the highest values of the whole plankton community. The highest amount was 85600 Ind./l.

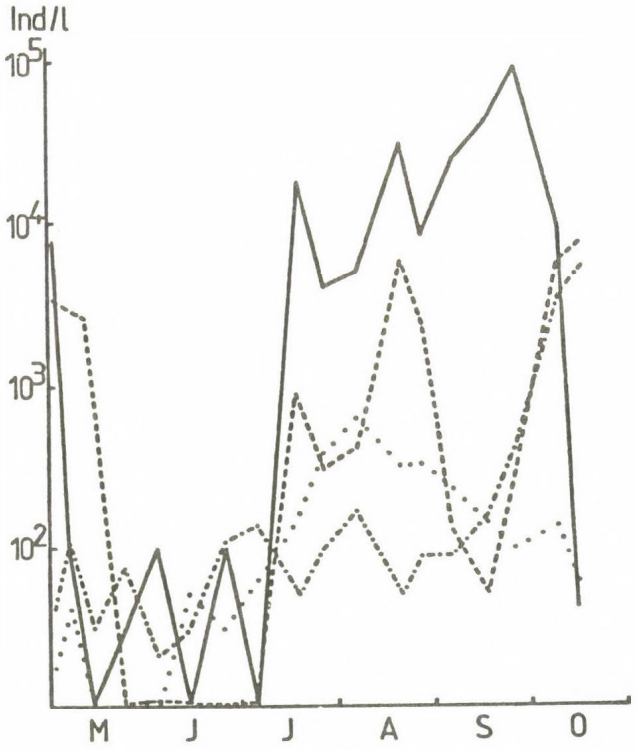

Fig. 1: Abundance of Copepodes (.....), Cladocera (-.-.-.-.-.-), Rotifers (----------) and Ciliates (——————) from May until October 1984 in Ehrendorfer Teich.

Comparing biomass of ciliated protozoa with biomass of the other zooplankton groups, we have to notice a contrary relationship. Though sometimes ciliate biomass is relatively high, standing crop of ciliate biomass can be observed at a very low level most time of the year.

Considering the development of ciliates in the course of the vegetation period not only increasing abundances but also an increasing percentage of total zooplankton biomass is to be stated. In ponds, which are filled with water and ice-covered during the winter period, also very high ciliate densities are to be found in early spring. Percentages up to 92 % of total zooplankton biomass can be observed.

Hunt & Chein 1983 report that protozoa can reach values up to 47 %, Pace 1981 observed ciliate biomasses between 15 and 61 %. In the mesotrophic Piburger See a percentage of 30 % was recorded at the highest (Schlott-Idl 1978). In a series of south-central Ontario lakes, ciliates comprise on the order of 5 to 10 % of the total zooplankton biomass (Gates 1984).

It can be supported that there is a relationship between the trophic state and the number and biomass of ciliates. A regression analysis between the annual mean of total phosphorus content and biomass of ciliated protozoa was calculated (Fig. 2).

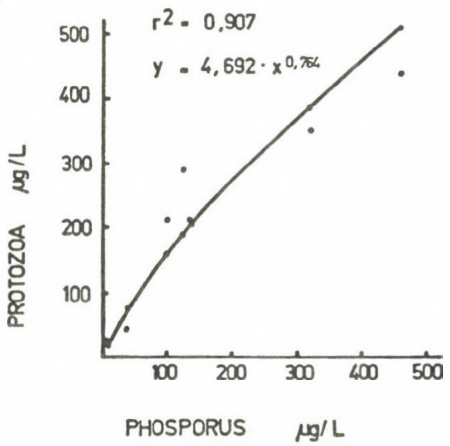

Fig. 2: Regression analysis between total phosphorus content and biomass of protozoa.

It must be ascertained that taxonomical problems were unfairly dealt with. Only few species could be identified. Altogether 30 different ciliates are differentiated. According to Corliss (1979) they can be classified into 13 Polyhymenophora, 10 Kinetophragminophora and 7 Oligohymenophora.

12 species could be identified. 18 are differentiated only at genus level. Codonella sp., Coleps spp., Didinium spp., Strobilidium spp., Strombidium spp., and Tintinnidium spp. are quantitatively important. Spirostomum teres, Spirostomum ambiguum and Loxodes striatus are present in high numbers in winter months.
Special regard is given to Loxodes striatus (Fig. 3).

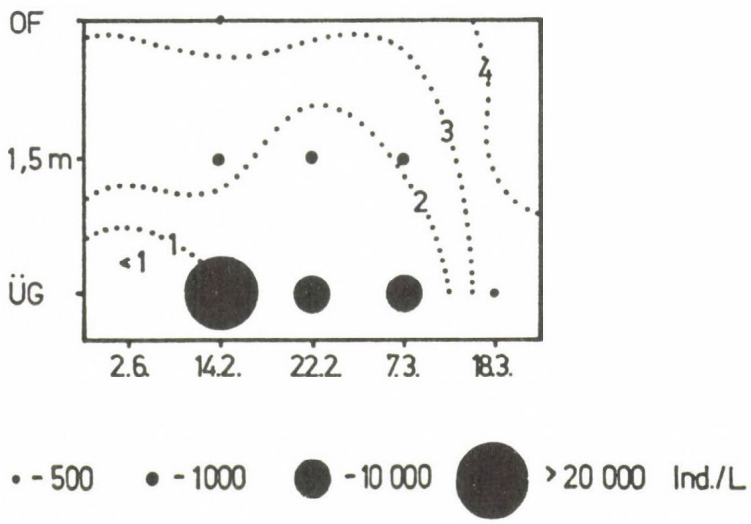

Fig. 3: Vertical distribution of Loxodes striatus in Steingrabenteich 2
(.....) Selected isolines of oxygen content.

It can be accepted that Loxodes sp. mainly is a member of the benthic protozoan community. Maximum densities were found an February 14th at the deepest layer, individuals are present throughout the whole water column. At the former sampling date a lack of oxygen could be observed. According to Goulder (1972) and Finlay (1981) ciliate migrations may be described as a retreat from increasing concentrations of reduced compounds. Loxodes sp. disappears simultaneously with increasing oxygen

concentrations. There is a clear relationship between the oxygen content and the occurrence of Loxodes sp. These data confirm the findings of Fenchel & Finlay (1984). They found that Loxodes sp. tends to swim up in anoxia or at very low O_2 tensions. At higher O_2 tensions they tend to swim down.

ACKNOWLEDGEMENTS

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I would like to express my gratitude to Mrs. Maria Futterknecht and Emmi Preißl for their help in the field and laboratory.

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CILIATES FROM SALINE LAKES IN SASKATCHEWAN

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The Saskatchewan saline lakes lie between 50° and 52° N - latitude and from 104° to 110° W - longitude. They occur in endorheic drainage basins and tend to be shallow. The maximum depth is 45 m. They range in area from 307 to 4.6 square km. All are located in lake basins of glacial origin.

They are closed lakes which receive water from snowmelt runoff in the spring and directly from precipitation. Ice covers the lakes for 5 - 6 month. Seasonal water temperatures range from zero to over 20 centigrade. The deeper lakes stratify in summer or they are meromictic. The range of pH is from about 7.8 to 9.3. The meromictic lakes are mesotrophic while the other lakes are eutrophic (HAMMER 1978 a, b, c).

The Maucha diagrams of the lakes in which ciliates were studied show a big variation in ion proportions and concentrations (Fig. 1). The lake waters tend to be dominated by sodium, magnesium and sulphate. Sodium is the dominant cation only in Deadmoose Lake while magnesium dominates the other six lakes. Sulphate is the dominant anion.

The salinities in September 1983 were the followings: Wakaw, Humboldt and Redberry with low degrees from 3.73 to 19.42, Waldsea, Big Quill and Little Manitou with salinities from 25.37 to 95.92.

In September 1983 samples were taken from benthic regions in the lakes, named above. I found 43 species of ciliates. The infraciliature was examined by impregnation techniques using silver nitrate and protargol (CORLISS 1953; WILBERT 1975). This was necessary to provide adequate diagnostics for species identification. Pictures are provided of species rarely observed or exhibiting morphological peculiarities.

16 of the 43 species, that is 39 %, are found in freshwater, for instance Ophrydium versatile. Most of them occur only at the lowest degree of salinity.

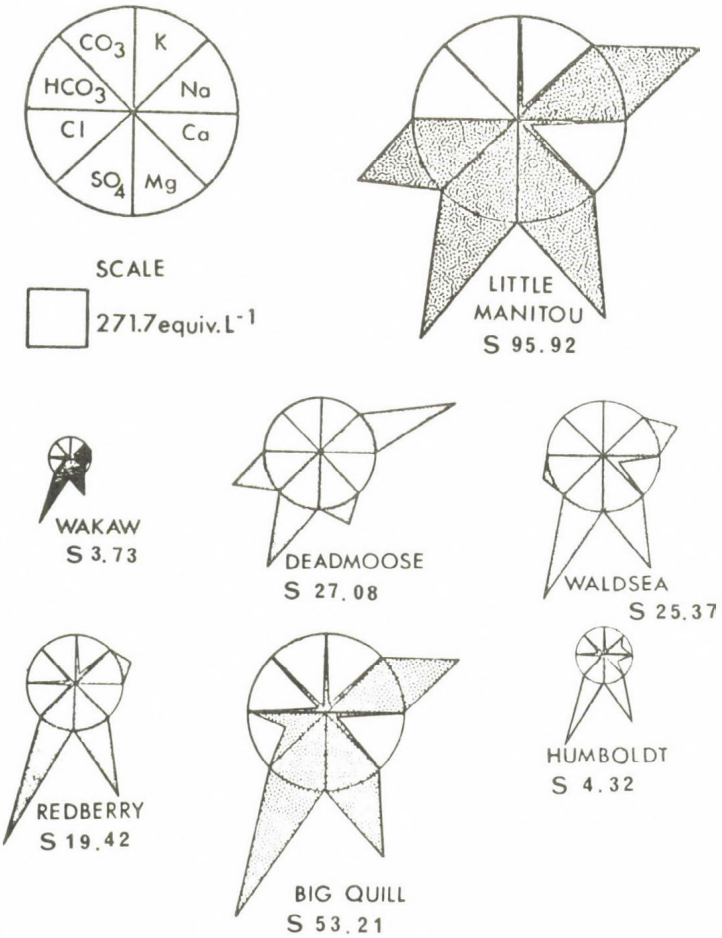

Fig. 1 Maucha diagrams showing the ion proportions and concentrations of the lakes in which ciliates were studied.

12 species are known as marine ciliates. That are 28 %. Orthodonella hamatus belongs to them (Fig. 2). It has been recorded first in the Mediterranean Sea. Its infraciliature was unknown. This species is totally ciliated, but the dorsal ciliature is less dense. The synhymenium on the ventral side of the beak is formed by paired kinetosomes. O. hamatus is considerably flexible. In contraction it measures 90 to 150µm. Extended, it has a length of until to 350µm. It feeds on diatoms.

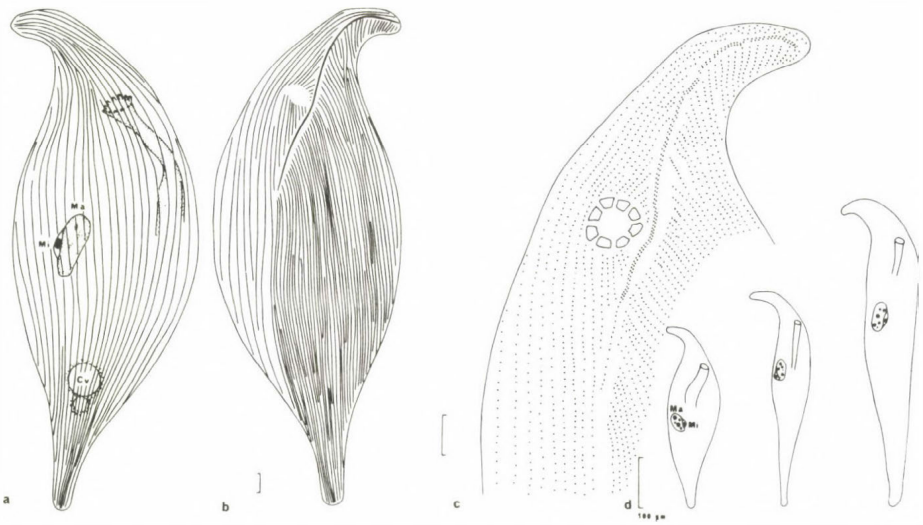

Fig. 2 Orthodonella hamatus GRUBER, a) dorsal and b) ventral arrangement of ciliary rows. c) anterior end, ventral aspect, showing infraciliature and the pharyngeal basket, d) change of size.
Cv, contractile vacuole; Ma, macronucleus; Mi, micronucleus.
Scales in all drawings indicate length of 10µm.

Other marine ciliates are Bakuella marina (Fig. 3) described by AGAMALIEV from the Caspian Sea and Strombidium styliferum LEVANDER. KAHL found it in the German Sea (Fig. 4).

10 of the 43 species (22 %) live as well in freshwater as in the sea. For example: Holosticha diademata (REES) KAHL (Fig. 5). KAHL found this species in all saline waters he investigated. It is also widely encountered in freshwater. I discovered the species in a very high salinity of 100 in the Solar Lake near Eilat (WILBERT 1981). In Saskatchewan I met it in the Waldsea with its salinity of 25,37 (Fig. 5).

Among the total number of 43 species remains a rest of 5 (11 %), only found in these salt lakes. To those species belong Holosticha geleii n.sp It is living in the Little Manitou Lake in a salinity of 95,92. 40 - 60 µm in length, this Holosticha is easily distinguished by its marginal cirri (Fig. 6). Normally, the marginal cirri follow the ventral margins. In this case the right marginal cirri start dorsally and take a diagonal course on the ventral side until they reach the transverse cirri (TC) at the caudal

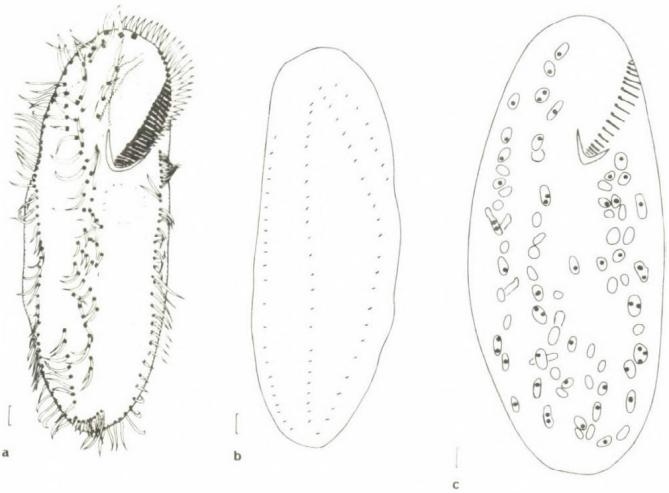

Fig. 3 Bakuella marina AGAMALIEV, overall view from living animals and protargol preparations. Ventral (a) and dorsal infraciliature (b), nuclei (c).

Fig. 4
Strombidium styliferum LEVANDER
Drawn from living animals and protargol preparations. Lateral aspect, showing the adoral zone of membranelles, macronucleus and various cytoplasmic inclusions. The arrows indicate an equatorial circle of simple cilia and a postequatorial kinety.

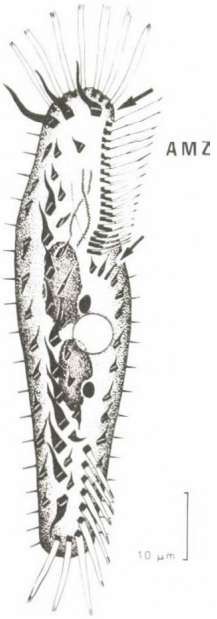

Fig. 5 Holosticha diademata (REES) KAHL
Ventral view of the infraciliature. Characteristics of the genus and the species are: the midventral cirri, that are two rows of ventral cirri in a zig - zag position, a row of marginal cirri on the left, which begins below the adoral zone of membranelles (AZM), four cirri close together at the right angle to the other cirri, and a wide gap in the AZM between the membranelles of the ventral and dorsal sides.

Table 1

Morphometric light-microscopic characterization of Holosticha geleii. Data based on protargol stained specimens. Legend: M, median; $\bar{x}$, mean; S, standard deviation; $S\bar{x}$, standard error of the mean; n, sample size. AZM, adoral zone of membranelles.

Character	M	$\bar{x}$	S	$S\bar{x}$	range	n
Body, length	50	51.08	5.71	1.65	42 - 63	12
Body, width	24	23.08	2.81	0.81	17 - 26	12
Number of macronuclei	2	2	0.0	0.0	2 - 2	12
Length of one macronucleus	7	7.36	2.06	0.62	5 - 10	11
Number of adoral membranelles	20	19.85	0.9	0.25	18 - 21	13
Length of the AZM	16	16.75	1.36	0.39	15 - 19	12
Number of dorsal kineties	3	3	0.0	0.0	3 - 3	10
Number of right marginal cirri	22	21.93	2.05	0.53	18 - 25	15
Number of left marginal cirri	20	20.47	1.81	0.47	18 - 24	15
Number of midventral cirri	9	8.55	0.69	0.21	7 - 9	11
Number of frontal cirri	4	4	0.0	0.0	4 - 4	10
Number of transverse cirri	5	5.00	0.5	0.17	4 - 6	10
Number of caudal cirri	3	3	0.0	0.0	3 - 3	10

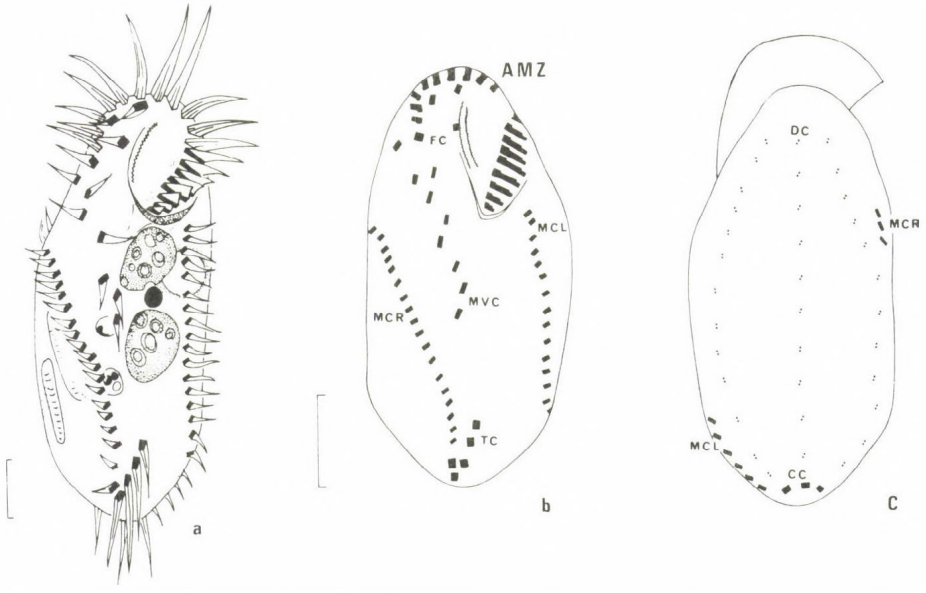

Fig. 6 Holosticha geleii n. sp.
Ventral aspect, drawing from combined observations of live and protargol stained animals (a), infraciliature of the ventral (b) and dorsal side (c).
AMZ, adoral zone of membranelles; CC, caudal cirri; DC, dorsal cirri; FC, frontal cirri; MCL, left marginal cirri; MCR, right marginal cirri; TC, transverse cirri.

end. The left marginal cirri start ventrally and cross the flank to the back. By that the body looks revolved. The nuclear apparatus consists of two oval macronuclei and one micronucleus. H. geleii feeds on diatoms.
Figure 7 brings some morphogenetic stages: They start with a proliferation

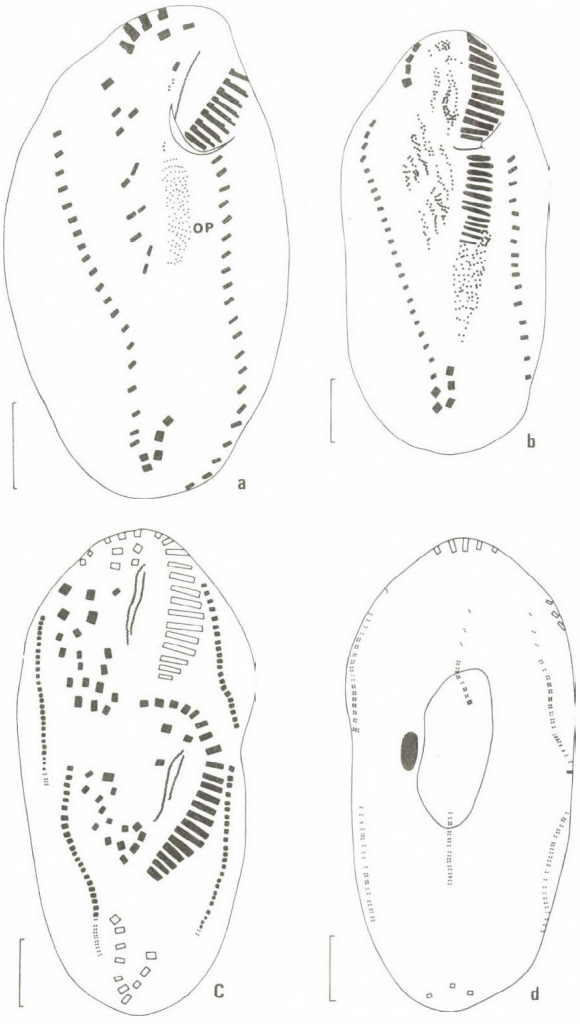

Fig. 7 Morphogenetic stages of Holosticha geleii n. sp

a) proliferation of the basal bodies for the oral primordium (OP).
b) ciliary fields (anlagen) from which midventral and transverse cirri of proter and opisthe will differentiate, and the newly forming AZM of the opisthe.
c) the frontal, midventral and transverse fields of cirri of proter and opisthe.
d) the caudal cirri arise from basal bodies at the posterior ends of the 3 dorsal kineties.

of basal bodies in order to build the oral primordium (OP). Then the midventral and frontal cirri form 5 anlagen as well in the proter as in the opisthe. Later on the 5 anlagen will differentiate in the frontal, midventral and transverse cirri of the daughter cells. The proter inherits the AZM of the parental cell.

On the dorsal side all three kineties develop the anlagen for the new caudal cirri.

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THE RELATIVE IMPORTANCE OF PROTOZOANS IN A BALTIC ESTUARINE ZOOPLANKTON COMMUNITY

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ABSTRACT

The proto- and metazooplankton at a station in the shallow eutrophic Darss-Zingst estuary (southern Baltic, S: 3-7 °/oo) was investigated for two years using live counts (droplet method) for protozoans and counts of sedimented fixed samples for metazooplankton. Protozoans significantly dominate the community numerically and account for most of the zooplankton biomass for the whole year, except for early summer, when copepods reach their maximum abundances and protozoans account for less than 30 %. Even though these are preliminary results they suggest that protozooplankton makes a very significant contribution to the matter flux of this eutrophic estuary.

INTRODUCTION

The knowledge of the importance of planktonic protozoans in natural aquatic ecosystems has increased, especially during the last decade, in marine (e.g. Bēers 1982) as well as in limnetic waters (e.g. Pace & Orcutt 1981). But until recently, only a few studies have been published on the role of protozooplankton in estuaries, even in the well investigated areas of the Baltic with respect to other biological components (e.g. Schwarz 1961, Biernacka 1963, Elbrächter 1970, Boikova 1984).

The ecosystem comprising the inner coastal waters south of the Darss-Zingst peninsula served as an object of modelling efforts for several years (cf. Vietinghoff 1984). Most functional groups have already been investigated, incl. benthic ciliates (cf. Scharf & Schnese 1984), but there is a lack for studies on protozooplankton.

MATERIAL & METHODS

Plankton samples were collected at station "Zingster Strom", Darss-Zingst estuary (S: 3- 7 °/oo; winter T_W: 0- 3 °C; summer T_W: 16- 24 °C; mean depth: 1.7 m) at intervals of a few days to 3- 4 weeks. 5l-Hydrobios samples were taken from a depth of 0.5 m. Protozooplankters were counted in living samples by a modification of Goulder's (1971) droplet method (in cases of extremely high abundances dilutions with particle free biotope water, <0.45 µm, were neccessary). For estimation of biovolume individuals were sorted into size classes. Flagellates were omitted, since, according to our present knowledge, they are largely autotrophic (Wasmund, pers. comm.). Small metazooplankters were counted in formalin-fixed sedimented samples under an inverted microscope, while larger zooplankters were counted from net samples (>56 µm)(unpubl. data by Schnese and Heerkloss). Since not all proto- and metazooplankton samples were taken at the same time, all data from each month (generally 3) were pooled together. For a description of the study area see Heerkloss et al. (1984).

RESULTS & DISCUSSION

Abundances of ciliates ranged from 2- 100 · 10^4 ind · l^{-1} and were highest for the smallest individuals (<30 µm) whereas middle size classes (50- 100 µm) dominate in terms of biomass. Seasonal variations in ciliate biomass are shown in Fig. 1, indicating higher values in spring and late summer, and lowest values in early summer. A similar pattern was found by Smetacek (1981) in Kiel Bight. Biomasses of protozoans are in the range of values reported from limnetic eutrophicated waters (cf. summary by Pace 1982). The spring maximum could be induced by high abundances of small phytoflagellates (Rhodomonas, Cryptomonas) which could serve as a food source for the dominating oligotrichs during that time. The summer maximum of protozoan biomass corresponds to high temperatures and maximum

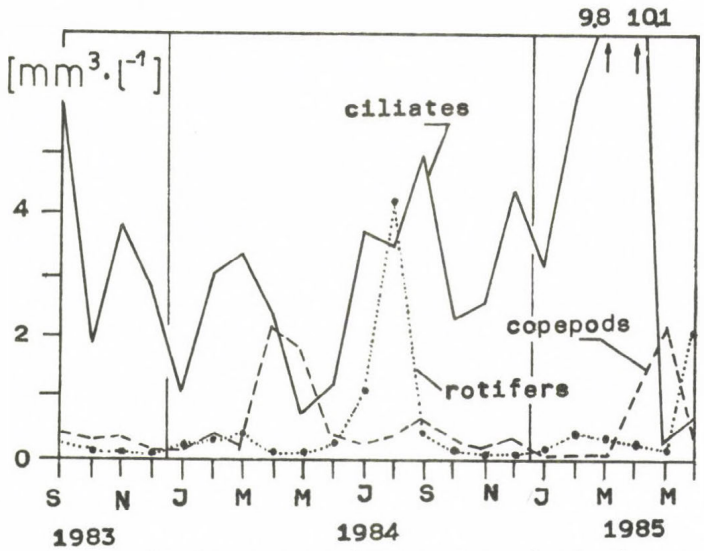

Fig. 1 Seasonal changes in biomasses of planktonic ciliates, copepods, and rotifers at station "Zingster Strom" in 1983-1985.

values of bacterial production (cf. Jost & Ballin 1984). During summer the dominating rotifer species which are known to be microconsumers (cf. Vietinghoff et al. 1984, Arndt et al. 1984), could be effective competitors of the ciliates. The sharp decrease in protozoan biomass in May is related to a very high biomass of the calanoid copepod Eurytemora affinis. Our own feeding experiments (^{14}C-method, unpubl.) and results by Berk et al. (1977) indicated that this copepod could be an effective consumer of ciliates. So for the interpretation of the late spring minimum of protozoans, in addition to qualitative changes in the protozoan community, predation could be discussed as a possible cause. For studies of causal relationships within the plankton community detailed qualitative studies are planned.

Fig. 2 indicates that ciliates are the dominant part of zooplankton biomass throughout the year, except for the period May-June. The annual mean biomass values of ciliates, rotifers, and copepods for 1984 were 2.70, 0.60, and 0.61 g fw $\cdot$ m^{-3}, respectively (other zooplankton groups were

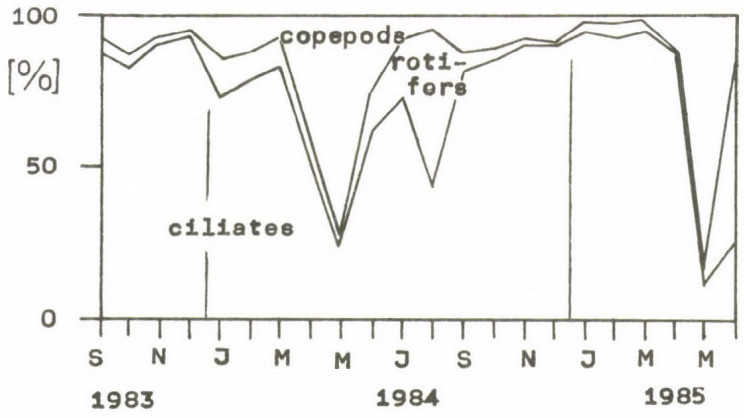

Fig. 2 Seasonal changes in composition of zooplankton biomass at station "Zingster Strom" in 1983-85.

apparently unimportant during the investigation period). Ciliates accounted for 70 % of zooplankton biomass, but due to high metabolic rates compared to metazooplankton, their importance in the matter flux could still be higher.

The possible impact of ciliates on bacterial production (Jost & Ballin 1984) and primary production (Börner & Kell 1981) was estimated by assuming a food ration of ciliates of about 100 % body weight per day (taking into account the dominance of small individuals this should not be an overestimation, Rassoulzadegan 1982). According to this rough estimation ciliates could consume about 50 % of annual bacterial production or 20-30 % of the sum of bacterial and primary production. Thinking on the food selectivity of ciliates (e.g. Fenchel 1980, Schönborn 1981) they should be able to control the dynamics of their preferred food sources. High feeding rates by ciliates during summer should be considered a possible cause for the dominance of large unconsumable phytoplankters in the estuary in summer and perhaps feeding pressure by ciliates rather than seasonal changes in metazooplankton composition explains the same phenomenon in many eutrophic limnetic waters.
The results also suggest that in the Darss-Zingst estu-

ary the short food chain, including phytoplankton, bacteria and protozooplankton, seems to be of major importance. The established high relative importance of protozoans confirm previous studies in marine (e.g. Beers 1982, Stegmann & Peinert 1984) and limnetic waters (Pace & Orcutt 1981).

Though these are only preliminary results, the overall significance of protozoans within the plankton community of the Darss-Zingst estuary underlines the neccessity of protozoological research in ecological studies of estuaries.

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SOME ASPECTS OF PLANKTON CILIATE ECOLOGY IN THE COASTAL REGION OF THE GULF OF RIGA

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INTRODUCTION

Data on plankton protists have a great importance in achieving a holistic understanding to marine ecosystems. In planktonic food chains ciliates form a link as they ingest the smallest food particles (Heinbokel, 1978) and are preyed on either by meso-, macro- or ichthyoplankton (Conover, 1982). Due to extremely high metabolism, the contribution of ciliates to the energy budget of the community is much greater than their fraction of the total biomass. The annual cycle and production of ciliates off the north-western coast of the Gulf of Riga were observed in a framework of common microzooplankton study in 1984.

MATERIALS AND METHODS

Samples were collected on three fixed stations situated on 10 m isobath two nautical miles off the coast-line. Distance between the neighbouring stations was 500 m. The stations were visited at 5-day intervals during the seasonal series of observation (7-17.01; 15-24.04; 11-29.07; 8-26.10).

The number (ind x m^{-2}) and biomass (mg x m^{-2}) of ciliates were determined by methods approved in the previous study (Andrushaitis, Martsinkevicha, in press). The division rate of ciliates was estimated on specimens from populations and main-

tained in nearly natural conditions as described elsewhere (Andrushaitis, 1984).

The total abundance of plankton ciliates was determined by small representatives of the suborder Oligotrichina, Bütschli throughout the year. The annual maximum of this group was observed in spring (April), when their density reached 137 x 10^6 ind x m^{-2}. The plankton was dominated by ciliates of genus Strombidium during this time with 30-80 µm cell size. There was also a considerable amount of larger forms such as S. viride (1.29 x 10^6 ind x m^{-2}) and S. conicum (1.66 x 10^6 ind x m^{-2}) (Fig. 1). A gradual decrease in oligotrichina number (down to 33 x 10 ind x m^{-2}) took place at the end of April. A second period of oligotrichina mass development occurred in late summer, reaching its maximum (63 x 10^6 ind x m^{-2}) at the end of July, with a predominance of individuals smaller than 25 µm. During autumn and winter periods the amount of oligotrichinae was rather stable, contributing to 6.3 x 10^6 and 2.5 x 10^6 ind x m^{-2}, respectively.

The annual maximum of tintinnid ciliates was registered in October. The highest density was observed in populations of Tintinnopsis beroidea Entz (0.67 x 10^6) and Tps. tubulosa (0.08 x 10^6 ind x m^{-2}). The tintinnids Helicostomella subulata and Tps. baltica were found occasionally in samples takes in autumn. In January and April Tps. beroidea and Tps. lobiancoe had loricae less than 70 µm long. During summer tintinnids were found rarely and did not play any considerable role in microzooplankton structure.

During all the seasons of 1984 we recorded high amounts of symbiotropic ciliate Mesodinium rubrum. In April and October Mesodinium numbers fluctuated within a range of 6.61 x 10^6-24.55 x 10^6 ind x m^{-2}, but in the July series it underwent a drastic fall from 223.90 x 10^6 to 1.96 x 10^6 ind x m^{-2}.

In the spring, summer and autumn series of observations, ciliates of the order Peritrichida Stein were found in the plankton. In spring they were represented mainly by free-living specimens of the genus Vorticella, but in autumn a number of parasitic peritrichidae appeared on Chaetoceros algae as well as on calanoid crustaceans of all developmental stages. The

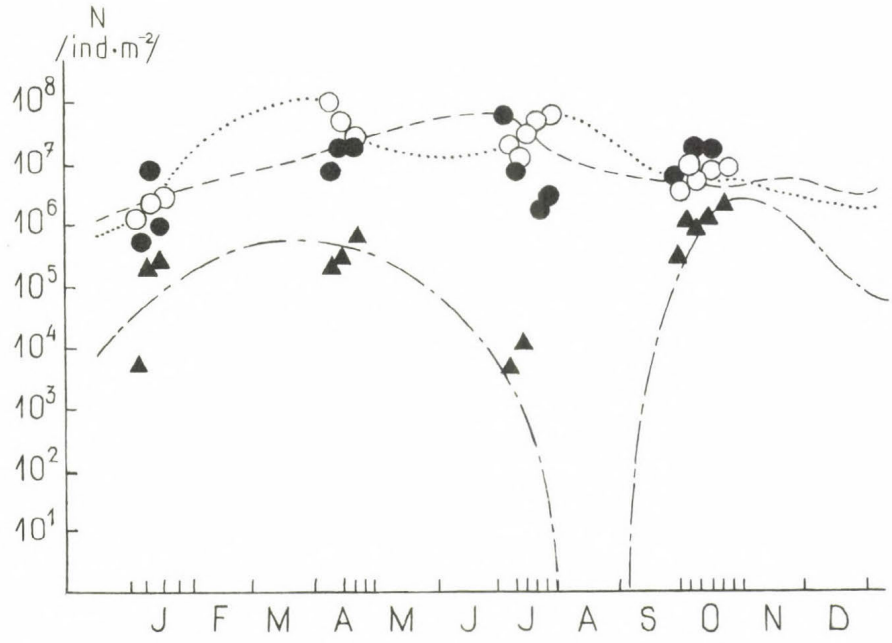

Fig. 1. Annual cycle of numbers of oligotrichina(o), mesodinium (●) and tintinnid (▲), 1984.

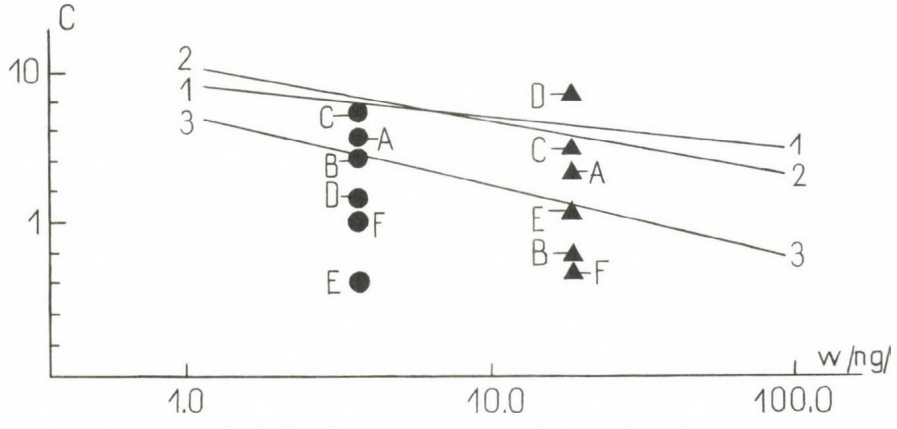

Fig. 2. Comparison between our values of diurnal specific production of Mesodinium rubrum (o) and Strombidium spp. (▲) (A - 07.83, B - 10.83, C - 01.84, D - 04.84, E - 07.84, F - 10.84) and the equations by Khlebovich 1974(1), Fenchel 1983(2), Klekowski and Tumantseva 1981(3).

latter group was particularly frequent giving 0.07 - 0.08 x 10^6 ind x m^{-2}.

The biomass of plankton ciliates ranged within 23.8-1944.8 mg w.w.m^{-2} during 1984. In the series of observations it averaged 27.16 (January), 919.21 (April), 362.02 (July), and 131.02 (October) mg x m^{-2}, giving 11.48, 31.80, 21.75 and 5.03 per cent, respectively, of the total microzooplankton biomass.

Production data of two most abundant ciliate groups are displayed in Table 1.

Table 1. Mean values of generation time (g), biomass (B) and diurnal production (P) of Strombidium spp. and Mesodinium rubrum in the Gulf of Riga

Form	Month	g(h)	B(mg x m^{-2})	P(mg x m^{-2} x day^{-1})
Strombidium spp.	01	69.30-32.57	22.40- 2.45	5.38- 3.40
	04	21.00- 1.72	624.84-945.89	493.62-841.50
	07	20.40- 8.70	49.25- 21.64	40.40- 41.85
	10	76.13-56.13	115.12- 85.01	25.33- 50.72
Mesodinium rubrum	01	43.31-23.55	6.80- 4.52	2.58- 4.09
	04	103.43-46.31	89.55- 32.16	14.33- 13.67
	07	60.03-29.14	281.41-427.34	78.80-211.80
	10	41.42-21.58	52.88 22.57	21.15- 24.87

DISCUSSION

A dominance of small oligotrichous ciliates in plankton of different regions of the World ocean was noted most clearly in studies carried out on live samples or applying special methods of fixation. A similar pattern of plankton structure was typical also for the Baltic Sea (Boikova, 1984), though single samples may give an approximate conception of ciliate distribution. The numbers of ciliates in three similar samples varied from 16 to 122 per cent in our research.

Comparison of ciliate relative production rate equalized to 20°C according to Krog's curve showed a considerable variability of these data concerning different ecological factors (Fig. 2). Periods of the highest ciliate standing crop not always correlate with the most rapid division rate. Thus, Mesodinium rubrum peaks were observed in the beginning of July, while the shortest generation times were obtained in the autumn-winter period. The occurrence of Mesodinium is typical of the cold season (Lindholm, 1978; Takahashy, Hoskins, 1978) and we can expect that it was controlled by predators in the investigated area. One of them may be rotifer Synchaeta baltica whose gut content had a bright red colour during that time.

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CORRELATIONS BETWEEN THE WATER SUPPLY AND THE MICROFAUNA OF VARIOUS TYPES OF SOIL

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The saying "Where there is water there is life" acknowledges the importance of water in the development and in the survival of life.

The water supply in some habitats is, however, limited. Soil protozoa can therefore be thought of as aquatic organisms but which are able to survive in soils because of the special and rapid adaptation to soil conditions. According to the classification of Varga /1962/, the protozoan fauna of soils may be categorized as a limnetic, hydrobiotic edaphon.

Protozoa of soils live within water which fills the pore spaces or in thin films of water on the surface of soil particles. According to Fehér and Varga /1929/, the activity of the microfauna is influenced both by the water supply and by temperature.

In cultivated ecosystems the water supply from rainfall is usually supplemented by irrigation, according to the types of crops being cultivated.

Helmeczi /1978/ has shown that the rate of cellulose and protein decomposition increases following irrigation. Horváth /1942-43/ and Lepsi /1951/ have also noted that irrigation may have a stimulatory effect on the protozoa.

MATERIALS AND METHODS

We have carried out our examinations of chernozem soils with and without irrigation and of alkaline soils. The chernozem soils have a medium nutrient content and their physical

structure is of a heavy clay. The soils were under maize culture.

Samples of alkaline soils were taken in the area of the Hortobágy National Park. In the higher areas here the soil is a medium meadow solonetz with Achilleo-Festucaetum pseudovinae plant associations. The lower areas have a degraded solodized soil with Poliuro-Plantaginetum tenuiflorae plant associations.

Samples were taken during dry period or during humid conditions, e.g. after rainfall or irrigation, from the upper 20 cms.

It should be noted that there are only 3 or 4 kilometres /as the crow flies/ between the two sites. The climatic conditions are consequently identical.

RESULTS

Figures 1 and 2 illustrate the changes in the total numbers of organisms and of the water content of both types of soil. The numbers of protozoa are strongly correlated with the water content.

During the early summer, the microfauna is initially dominated by flagellated protozoa. The numbers of ciliates and amoebae were low, and in regions without irrigation were greatly reduced as a consequence of lower water content.

Irrigation results in a greater number of organisms and there is a greater number of species. After irrigation, the water content of the soil falls as a result of percolation and evaporation /to 28.9 volume%/. The numbers of protozoa also change. This is in part a result of the water supply but is also a result of changes in the composition of the fauna. There was hardly any change in the numbers of ciliates, and their continued feeding activities might have led to a decline in the total counts of organisms.

The heavy rainfall in June /76.5 mm/ and in July /129.1 mm/ significantly affected the microfauna. The activity of the protozoa was elevated, particularly in the chernozem soil. There was a sudden increase in the total numbers of indivi-

duals. Amoebae and flagellates were present in highest numbers but ciliates were also relatively numerous. After the rain, the water content of the chernozem soil decreased rapidly.

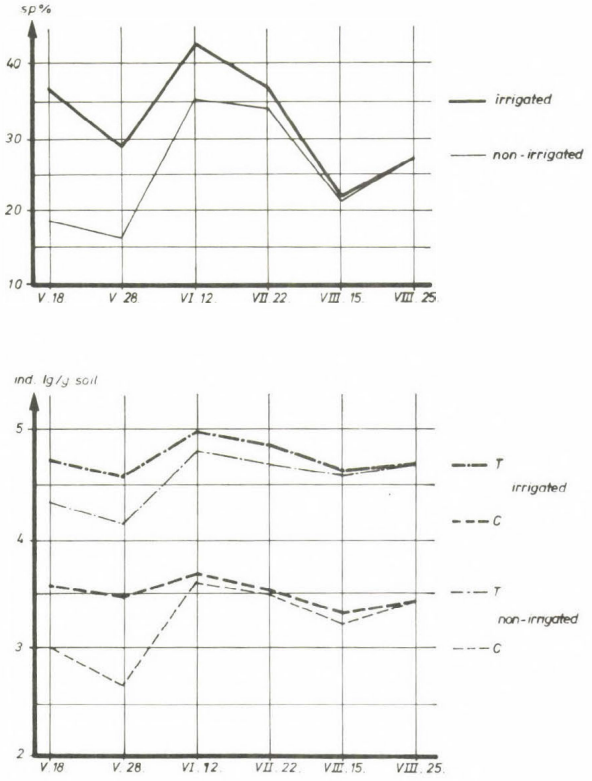

Fig.1. Quantitative changes in the moisture content and protozoan fauna in the chernozem soil.

In alkaline soils, the numbers of protozoa /see Fig.2/ were usually an order of magnitude lower than in chernozem soils. The number of species found was also not as great. In early summer the microfauna was dominated by flagellates. The numbers of amoebae and ciliates was low. In the solodized solonetz soils, ciliates were not detected.

The chemical and physical characteristics of the alkaline soils appear to hinder the development of a diverse and numerous microfauna.

In both soil types, flagellates and amoebae were dominant, although the ciliates were also relatively numerous.

Thus, one might say that these "lower" organisms may remain active, even when the water content of the soil is relatively low. In spite of being extraordinarily adapted, the ciliates as"higher" organisms, become active only when the water content is high.

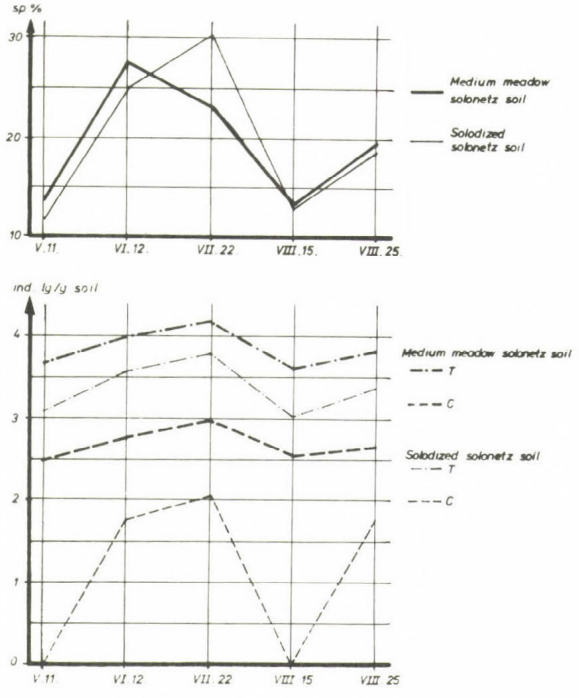

Fig.2. Quantitative changes in the moisture content and protozoan fauna in the alkaline soil.

During the drier period in August, the decrease in the soil water content exerted an unfavourable influence on the soil protozoa. The rapid warming of the soil, the thinning of the water film, the increase in the salt and CO_2 content stimulated encystment.

Amoebae and ciliates were found only in the chernozem soils with a better soil structure and with a higher water content. Species included A.terricola, Colpoda inflata, Chilodonella cucullulus, Spathidium spathula. The improved humidity resulting from irrigation and rainfall increased the numbers of individuals and the variety of species. This was

most evident after 3 or 4 days. The ciliates Euplotes novemcarinatus, Halteria grandinella, Prorodon teres, Colpoda cucullus, C. steini and Cyclidium citrullus were particularly numerous.

Because of the extreme soil conditions, species with a wide ecological range are encountered most commonly. These are usually species of Colpoda such as C. cucullus, C. inflata and C. steini. Organisms belonging to Halteria grandinella, Spathidium spathula and Uroleptus halseyi could also be encountered. With a decline in the water content, the numbers of organisms and those of ciliate species became lower.

Changes in the moisture content of soils

Date 1981	Chernozem		Alkali	
	non-irrigated	irrigated	medium meadow solonetz soil	solodized solonetz soil
V. 11	-	-	13.8	11.7
V. 18	18.6	36.7	-	-
V. 28	16.3	28.9	-	-
VI. 12	35.4	42.5	27.5	25.2
VII. 22	34.2	36.8	23.2	30.3
VIII. 15	21.5	21.7	13.2	12.8
VIII. 25	27.4	27.4	19.7	18.7

SUMMARY

In chernozem soils populations contain more species and more individuals as a result of better supply of O_2, the higher nutrient content, the variety of nutrients, the larger pore spaces and capillaries. It is here that the ciliates are relatively more abundant. In the alkaline soils, the ciliates make up a smaller fraction of the total number of organisms. This may be explained by the small size of the soil pores and by poor water balance.

We have shown that certain numbers of the microfauna, especially flagellates and amoebae, may be active even at water contents as low as 11 to 18 volume%. The ciliates usu-

ally encyst at water contents less than 13 volume%. This phenomenon is more evident in chernozem soils than in alkaline soils. Repeated increases in the water content lead to a rapid growth of the microfauna.

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COMPARISONS BETWEEN THE CHARACTERISTICS OF THE PRODUCTION OF TESTACEA (PROTOZOA, RHIZOPODA) IN DIFFERENT FORMS OF HUMUS

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1. INTRODUCTION

This paper deals with the comparison between production, mortality, abundance, and the time of decomposition of empty shells of Testacea in mull, moder, raw humus, and aqueous humus samples (suspensions). Comparisons of the production of Testacea and their parameters in various kinds of humus are still difficult because of the limited data. Nevertheless, there are evident indications that the kind of humus influences testacean mortality (Foissner and Adam 1981; Lousier 1984a, b; Schönborn 1975, 1982). Furthermore, the rate of mortality has an effect on production and abundance.

2. THE SOILS

The present results refer to the following soils:

a. Mull. C/N 21.8; p_H (H_2O) 7.0. Geological subsoil: shell lime. Vegetation: Ash (Fraxinus excelsior), Acer platanoides, Aesculus hippocastaneum, Cornus sanguinea, Aegopodium podagraria. Investigated: F (fragmented litter) and H (humus) layer. Jena, GDR.

b. Moder. C/N 21.0; p_H (H_2O) 5.1. Geological subsoil: shell lime. Vegetation: Beech (Fagus silvatica). Investigated: F and H layer. Jena, GDR.

c. Raw humus. C/N 26.1 p_H (H_2O) 4.8. Geological subsoil: argillaceous slate. Vegetation: Spruce (Picea abies). Investigated: litter (needle layer) and H layer. Plothen (Thuringia) GDR.

3. METHODS

Production, mortality, abundance, and the decomposition of empty shells were estimated as described by Schönborn (1975, 1978, 1982). The production chambers used (length 7 mm, diameter 3 mm) were sealed by two membrane filters (10 µm pores). The soil volume of the chambers averaged 0.05 ml (wet wt.). Observations were made weekly. The generation times were both measured in the chambers and calculated by the formula described by Lousier (1984b).

4. RESULTS

4.1. Abundance and production of Testacea in mull, moder, raw humus, and suspensions

The abundance curves, compared with the curves of production, are shown in Fig. 1 (mull) and Fig. 2 (raw humus, H layer). The curves indicate significantly lesser density in the raw humus (H layer) than in mull. In mull the abundance curve at first oscillated, but it stabilized from summer to winter at a high level. Similar patterns of population fluctuations were recorded by Lousier (1984a, b) in an aspen woodland soil. In raw humus the density oscillated throughout the year. This phenomenon was also reported by Couteaux (1976). Production followed a more discontinuous course than abundance. The suspensions show for many species a high abundance, with high longevity of individuals and a low production. In the litter (needle layer) of the raw humus, production and abundance were found to appear in high and short peaks (Fig. 3). Therefore production and abundance are higher than in the underlying soil

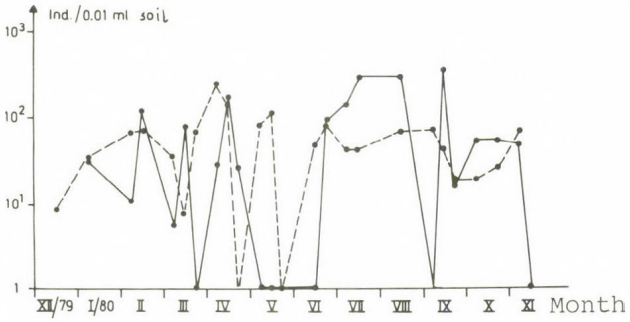

Fig. 1. Density and production of Testacea in mull. The production values apply to the time, which in each case is the last point marked. The distance between two marks within a month amounted to nearly 7 - 8 days. (After Schönborn 1982.) ---- Abundance; —— production

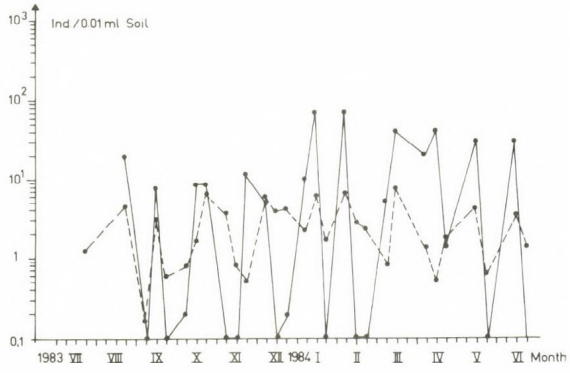

Fig. 2. Density and production of Testacea in raw humus (H layer). (Points and curves, see Fig. 1)

and in moder (see also Couteaux 1972; Geltzer et al. 1980; Schönborn 1962). In the raw humus there are two clearly distinguished types of production-abundance relationships: a retardative (H layer) relationship and a productive one, characterized by extreme peaks (L layer).

Testacean density shows substantially smaller differences between litter and humus layer in mull as well as in moder than in raw humus. In the needle layer Euglypha ciliata reached a high density only two times in a period of 2 weeks.

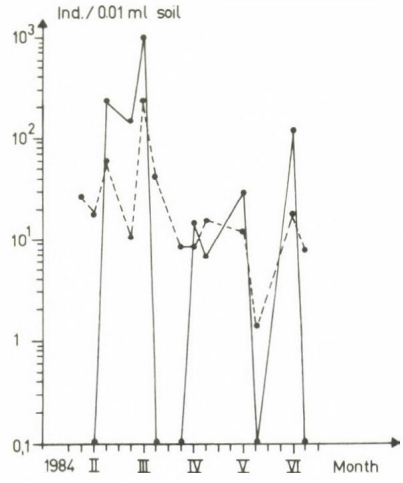

Fig. 3. Density and production of Testacea in raw humus (L layer, needles). (Points and curves, see Fig. 1)

In this time the production was nearly zero. Empty shells (mortality) were found towards the end of this period. This type of production-mortality-abundance relationship was found frequently in suspensions. The information in Table 1 can be summarized with the following comments. In the H layer of the raw mull to raw humus production-abundance and generation times decrease, but the production-abundance quotient $P/\bar{A}$ tends to increase. In the needle layer production and abundance are higher than in moder, caused by Corythion dubium, an excellently adapted species. But also in mull the production is lower than in the needle layer. In the needle layer populations peaked in short time periods. In the growth phase the generation time can be very short, but in periods of low densities the generation times were 7 days and more. In the H layer of raw humus the low density is connected with relatively high production and high turnover. In suspensions the same species show a high abundance, a low production and a relatively greater longevity of individuals. In mull many species appeared with high production and abundance, but the turnover was substantially slower than in raw humus.

Table 1. Comparisons of the characteristics of testacean production in mull, moder, raw humus, and suspensions (± standard error)

Kind of humus	Mean abundance $\bar{A}$ (0.01 ml^{-1})	Annual production P(ind.·0.01 $ml^{-1}yr.^{-1}$)	Mean generation time $\bar{T}$ (days)	$P/\bar{A}$
Mull (H layer)	60.8 ± 33.4	1881	6.34 ± 2.8	30.9
Moder (H layer)	30.0 ± 16.5	450	4.46[1] ± 1.52	15.0
Raw humus				
H layer	2.1 ± 2.1	254	1.84 ± 0.71	120.9
L layer	31.7 ± 54.3	3620[2]	1.39[3] ± 0.63	114.2
			8.8 ± 1.4	?
Suspensions[4]	142.8 ± 39.4	1577[5]	10.6 ± 9.0	11.0

[1]After Lousier (1984a,b): aspen woodland soils
[2]Measured 1508 ind. · 5 $months^{-1}$
[3]Only in the phase of growth
[4]From raw humus
[5]Measured 657 ind. · 5 $months^{-1}$

4.2. The role of mortality in testacean populations

The mortality essentially determined the different relationships between production and abundance. However, the total mortality for a long time (e.g. month or year) in all habitats is relatively constant, and amounted to about 100% of the production. Nearly all individuals which are produced in a year die in the same year.

The different relationships between production and abundance are probably due to the distribution of the mortality throughout a whole period of growth. There are two forms of mortality distribution: a homogeneous distribution and an increase at the end of the growth. The homogeneous distribution indicates that many individuals die a short time after cell divisions. Such an "early mortality" is independent of density.

I	II
P = 8	P = 14
$\bar{A}$ = 1.5 [1.8]*	$\bar{A}$ = 5.5 [3.7]*
P/$\bar{A}$ = 5.3 [4.4]	P/$\bar{A}$ = 2.5 [3.8]
G = 5	G = 4
T = 1 day	T = 1.25 days
Control: 5 days	Control: 5 days

Fig. 4. Graphic models to explain the retardative (I) and productive (II) PMA-type. P: Production, $\bar{A}$: mean abundance, T: generation time. $\bar{A}^*$ as total density of shells divided by number of generations (G)
● full shells (live individuals); o empty shells (dead individuals)

The two types of mortality distribution are presented in the form of graphic models (Fig. 4). The populations in the H layers of raw humus are characterized by type I (early mortality), those in mull by type II. The high early mortality in raw humus compensates for short generation times; however, production and abundance do not reach the values found in mull. Observations at the end of a growth period of type II automatically show a high P/$\bar{A}$ quotient, but most observations are carried out before the end. In type II a high abundance extends the generation times. Production decreases and mortality increases. Generation time can be dependent on density. A high mortality reduces the density and increases the turnover. An increase of density reduced the turnover.

4.3. Decomposition of the empty shells

The decomposition of the empty shells is also dependent on the kind of soil. In raw humus the decomposition of shells is substantially slower than in moder and mull. The values are: Mull: Half-time of decomposition 6 days, total time of decom-

position 27 days. Moder: Half-time 28 days, total 85 days. Raw humus: Half-time 48 days, total >160 days. All values refer to experiments in situ during moist weather in April. Lousier and Parkinson (1981) recorded in an aspen woodland soil in experimental cultures a shell disappearance rate of 42 - 97% in the first week.

5. DISCUSSION

The production-mortality-abundance relationships (= PMA types) are dependent on the kind of humus and other environmental conditions. It is not clear whether the PMA types are also caused by genetic factors. In mull Centropyxis aërophila var. sphagnicola and Phryganella acropodia belong to type II (= productive type): in raw humus (H layer) the same species belong to type I (retardative type) (Lousier 1984b; Schönborn 1983). In contrast with the natural soils, the individuals of the named species in suspensions show a greater longevity (high abundance, low production) (= optimal type). In mull Euglypha ciliata and Corythion dubium represented type I, in the needle layer, however, type II (Schönborn 1983). The optimal type seldom occurs in soils. Lousier (1984a, b) found that the densities of empty shells in moder parallel those of the densities of live animals at a high level. Such a "state of shells" indicates the productive PMA type. From Figs 5 and 6 it can be seen that the chosen species in mull, raw humus, and suspensions represent different types of PMA relationships.

Lousier (1984a, b) reported considerable differences of generation times in the L, F, H, and Ah layers. This result also shows the exogenous influence upon the PMA relationships. Generally, four PMA types can be distinguished (Fig. 7). The sporadic type (Fig. 7D) shows a low production and abundance. The clones become extinct very quickly. This PMA type occurs in all soils.

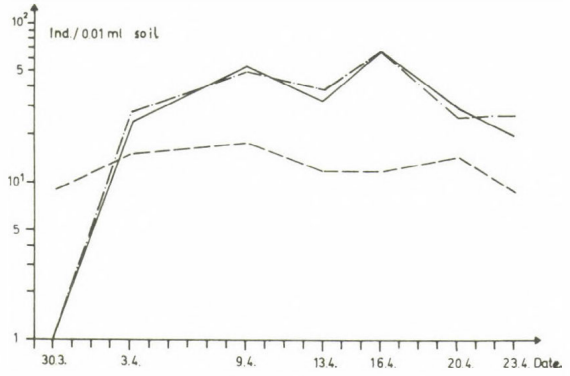

Fig. 5. PMA relationships in Trinema enchelys in mull. Productive (II)-type. (After Schönborn 1983.)
—— Production; -.-.- mortality; --- abundance

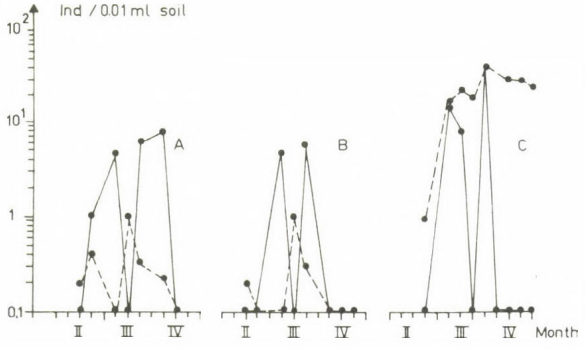

Fig. 6. PMA relationships in raw humus (H layer) and suspensions. A = Trinema enchelys, B = Phryganella acropodia. Both in raw humus; retardative (I) types. C = Euglypha ciliata in suspensions. Optimal type (Curves see Fig. 5)

6. SUMMARY

In the H layer of the raw mull to raw humus the production and abundance of Testacea decrease and the early mortality (= the density-independent mortality a short time after cell division) of the species increases. In the litter (= needle layer) of the raw humus in a sprucewood, abundance and production are higher than in moder, caused by Corythion dubium, an

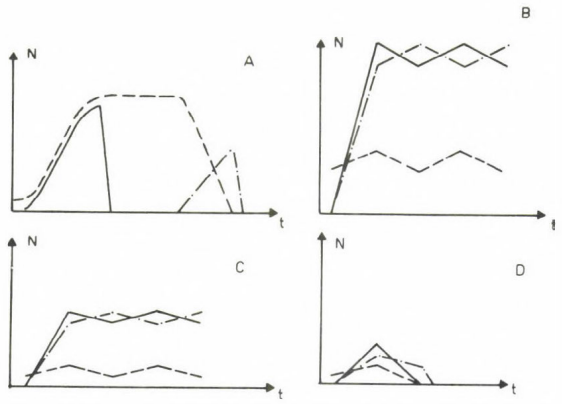

Fig. 7. The PMA types, schematic. A: Optimal type. B: Productive type. C: Retardative type. D: Sporadic type (Curves, see Fig. 5). (After Schönborn 1983)

excellently adapted species. In raw humus the decomposition of empty shells in substantially slower than in moder and mull. In suspensions the longevity of individuals and their abundance increase and production decreases. The average generation time, measured in the H layer of the raw humus, was often shorter than in moder, mull, and suspensions. Production, mortality, generation times, and decomposition of empty shells are dependent on the kind of humus and other environmental factors.

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THE DECAY OF EMPTY TESTS OF TESTATE AMOEBAE (RHIZOPODA, PROTOZOA)

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INTRODUCTION

In recent years evidence has accumulated that protozoa play an important role as predators of microorganisms. In microcosm experiments (Anderson et al. 1978) the stimulating effects of Protozoa on nutrient cycling and energy flow has been demonstrated. Together with naked amoebae, testate amoebae are the major protozoan groups in soils (Lousier & Parkinson 1984; Meisterfeld, contribution this volume; Schönborn 1982). Because of their test, which makes estimation of death rates possible, they are very well suited for measuring secondary production. The accuracy of these estimates however, greatly depends on the pace with which empty tests disdisappear from the soil (Lousier & Parkinson 1981).

MATERIALS AND METHODS

To study test decomposition, F-layer material of a 130 year old beech forest in the Solling area (site description in Meisterfeld, 1980) was moistened with MERCK Standard I nutrient broth (0.2%) to stimulate growth of bacteria and finally of testacea. The microcosms were set up in 50 ml beakers and oven dried at 80 ^{o}C for 5 h in order to kill all testate amoebae whilst allowing spores of microorganisms to survive. Previous experiments had shown that higher temperatures resulted in significantly higher losses and faster dis-

appearance of shells (Lousier & Parkinson 1981). After drying, the soil was remoistened with distilled water. Two series of experiments at 10 and 20 ^{0}C were set up. The moisture content varied between 74 and 78%. After 3, 7, 10, 20, 30, 42 and 97 days 5 microcosms were randomly chosen and destructively sampled. The number of remaining tests was estimated by direct microscopic counting. The decay data were fitted to the following four mathematical models by a least square regression using the Tektronix program package PLOT 50.

(1) $y = a + bt$ (2) $y = ae^{bt}$

(3) $y = 1 / (a + bt)$ (4) $y = a + b\ln(t)$

RESULTS

The moistening with nutrient broth resulted in a rapid increase of testacean densities. Small species of Euglyphidae like I. complanatum had 15 times higher abundances than at the beginning of the experiment. The tests disappeared rapidly (Tab.1). In our experiment at 20^{0}C, 40% on average are lost within 7 days. The significantly slower disappearance at 10^{0}C demonstrates that decomposition is stimulated by higher temperatures. Most of the species showed two different decay phases. After very rapid decomposition during the first 10 days the process slowed down distinctly. Patterns of test disappearance for the species whose tests are composed of sediment particles and those from the platelet type were not significantly different, although the Nebela species decay slower. The decay patterns of the different species are further characterized by fitting to mathematical models (Tab. 2). The goodness of fit is measured by r^2. In most cases the data are best fitted by the logarithmic model. The exponential model is the best for some decay patterns at 10^{0}C only.

DISCUSSION

The decay of empty tests is substantially slower than

Table 1. Percentage of empty tests remaining.

Species	Temp. (°C)	Days from beginning 3	7	10	20	30	97
Centropyxis sylvatica	10	83.2	79.6	53.1	36.3	44.2	39.8
	20	56.6	58.4	44.2	29.2	36.3	26.5
Cyclopyxis eurystoma	10	92.0	87.2	76.0	76.0	70.4	32.8
	20	88.8	56.8	59.2	46.4	66.4	14.4
Trigonopyxis arcula	10	94.3	76.5	72.6	68.7	60.4	57.0
	20	71.7	45.2	43.9	36.1	34.8	30.4
Hyalosphenia subflava	10	124.3	85.7	97.1	101.4	57.1	41.4
	20	88.6	57.1	55.7	55.7	41.4	38.6
Nebela collaris	10	106.3	99.2	93.8	95.3	84.4	72.7
	20	103.1	63.3	57.8	60.9	66.4	46.9
N. militaris	10	121.6	107.2	96.4	91.9	65.8	74.8
	20	91.0	64.0	70.3	59.5	49.5	40.5
N. tincta	10	112.4	103.4	89.9	80.9	82.0	44.9
	20	104.5	61.8	58.4	48.3	55.1	23.6
Phryganella acropodia	10	81.4	67.7	66.3	58.9	45.6	22.9
	20	57.0	45.3	40.2	30.7	26.9	10.8
Euglypha strigosa	10	109.3	100.7	94.3	105.7	95.7	70.7
	20	108.6	60.0	60.7	62.1	61.4	34.3
E. rotunda	10	96.8	77.0	74.6	70.7	50.9	37.0
	20	83.7	55.3	51.7	41.1	29.9	26.0
Assulina muscorum	10	92.8	74.9	65.6	62.7	65.9	46.4
	20	80.3	44.3	39.2	41.3	33.1	17.9
Corythion dubium	10	85.4	75.2	75.7	71.1	59.1	31.5
	20	68.1	62.0	58.4	49.3	39.0	16.1
Trinema complanatum	10	88.2	69.8	67.2	66.6	48.2	37.8
	20	79.7	59.2	52.1	32.6	28.6	23.0
T. lineare	10	90.5	72.8	63.5	62.7	44.6	24.6
	20	69.7	60.0	52.6	36.4	29.8	15.0
all tests	10	90.4	74.0	68.8	66.4	50.5	33.1
	20	74.2	57.2	51.4	37.9	32.1	19.3

Table 2.
The fit of experimental decay data to different mathematical models. Goodness of fit is measured as r^2 (an index of determination). The best fitted models are underlined.

Species	Temp. (°C)	Model 1	2	3	4
Cent. sylvatica	10	0.316	0.324	0.274	0.620
	20	0.393	0.434	0.391	0.947
Cycl. eurystoma	10	0.938	0.959	0.911	0.696
	20	0.688	0.764	0.707	0.768
Trig. arcula	10	0.537	0.577	0.596	0.872
	20	0.362	0.392	0.353	0.943
H. subflava	10	0.661	0.721	0.733	0.432
	20	0.456	0.505	0.498	0.876
N. collaris	10	0.786	0.817	0.840	0.531
	20	0.414	0.450	0.440	0.694
N. militaris	10	0.460	0.492	0.499	0.362
	20	0.552	0.623	0.630	0.838
N. tincta	10	0.894	0.903	0.877	0.478
	20	0.621	0.702	0.795	0.760
Phr. acropodia	10	0.754	0.860	0.949	0.885
	20	0.508	0.615	0.797	0.999
E. strigosa	10	0.751	0.759	0.758	0.329
	20	0.559	0.610	0.648	0.695
E. rotunda	10	0.754	0.827	0.884	0.787
	20	0.495	0.567	0.550	0.909
A. muscorum	10	0.620	0.671	0.704	0.865
	20	0.489	0.563	0.626	0.912
Cor. dubium	10	0.870	0.927	0.956	0.818
	20	0.703	0.820	0.940	0.943
T. complanatum	10	0.668	0.741	0.787	0.880
	20	0.504	0.581	0.557	0.917
T. lineare	10	0.763	0.858	0.945	0.838
	20	0.610	0.732	0.870	0.960
all species	10	0.756	0.836	0.902	0.853
	20	0.571	0.671	0.743	0.961

previously reported. At 20 °C 36 - 55.7% and at 10°C maximally 32.3% disappeared within 7 days. Lousier & Parkinson (1981) found losses between 74 and 98% in the first week. Schönborn (1982) studied test decomposition in mull and moder. In moder only 7% were lost after two weeks, while in mull 50% disappeared during the first 6 days.

Test decomposition is a biological process. So it is not surprising that higher temperatures stimulate decay. The reason why Lousier & Parkinson (1981) could not detect a positive correlation between temperature and pace of disappearance are perhaps because of the extremely high losses during the first days, which probably mask the differences between the different treatments. In our experiments decomposition is a microbial process, due to the fact that larger members of the soil meso- and macrofauna are missing. In situations where for example earthworms are abundant test disappearance can also be caused by enzymatic attack and mechanical stress when passing the gut.

Microbial activity depends largely on the availability of suitable substrates to keep the microorganisms metabolically active. The two different decay phases in our experiments probably reflect periods of significantly different microbial communities. After partial sterilisation and remoistening, most bacteria, fungi and other soil organisms have been killed. So a large amount of rapidly usable substrates is available to the microflora which is developing rapidly. The resulting flush of CO_2 indicates the high microbial activity. This is a well known fact and is used to measure microbial biomass in soils (Jenkinson & Powlson 1976). Within few days the easy to use substrates are depleted and activity of bacteria and fungi is only a small fraction of the peak values and the decay of empty tests is very slow.

In our experiments there is no simple relationship between the type of test composition and pattern of test disappearance. None of the species shows a linear decay. The Nebela species whose tests are composed of idiosomes of captured euglyphid testaceans and mineral particles often

embedded in a mass of organic cement decompose slowly. Unfortunately, these species were not abundant (0.9 - 1.5%) in our material and therefore confidence intervals are large and the fit to the different models of decay is bad. On the other hand, Phryganella acropodia, which has the same test structure, decomposes very rapidly, a result that corresponds well to the findings of Lousier & Parkinson (1981).

The influence of test disappearance on the calculation of secondary production is high. Using the data of this experiment together with estimates of testacean densities in a beech forest soil on lime near Göttingen (FRG), the decay term accounts for 34% of the production. During several intervals this fraction exceeds 50%.

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THE IMPORTANCE OF PROTOZOA IN A BEECH FOREST ECOSYSTEM

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INTRODUCTION

In the last years evidence has grown that protozoa make an important contribution to energy flow and nutrient cycling in terrestrial ecosystems. For an evaluation of the role of protoza in such systems essentially three different approaches have been followed.

In the field, the sizes, fluctuations and production of the protozoan populations are described and correlated with abiotic and biotic factors like rain fall or microbial biomass (Clarholm 1981, Foissner & Adam 1981, Lousier & Parkinson 1984 and Schönborn 1982).

Additional information concerning the controlling factors were obtained for example by Lousier (1974) in field experiments.

In a series of simplified, gnothobiotic soil microcosmos experiments Coleman et al. (1977, 1978), Anderson et al. (1978), Bryant et al. (1982) and Woods et al. (1982) demonstrated that amoebal grazers can reduce bacterial populations by up to 80% and that in grazed systems respiration and mineralisation of N and P were significantly higher.

In most cases, these studies were restricted to certain protozoan groups and information on other biotic components is lacking.

The present study is part of a multidisciplinary beech

forest project near Göttingen (FRG) and was designed to give information on the relative importance of different protozoan taxa (testate amoebae, naked amoebae and flagellates) and to allow comparisons with other relevant groups of soil organisms.

MATERIALS AND METHODS

Site

The study area is a 120 years old submontane beech forest in the east of Göttingen (Lower Saxony). The bed rock is shell lime stone from which a mull rendzina developed. The understory is a herb layer with Mercurialis perennis in the main.

Protozoa

At each sampling date, 12 replicate soil cores were taken and split into the 0 - 3 cm and 3 - 6 cm layers and then pooled. Testate amoebae were evaluated during the period from April to October 1981 (0 - 3 cm layer), naked amoebae from October 1981 to August 1983 (0 - 3 cm) and flagellates from November 1981 to August 1983. From February 1982 amoebae and flagellates were sampled additionally in the 3 - 6 cm layer in weekly intervals, too. Data for testacea were converted to annual estimates by assuming equal densities and production during the evaluated 6 months and the rest of the year. To have an approximate estimation of mean population size and production in the 3 - 6 cm layer, the relation of the corresponding data of naked amoebae in 0 - 3 and 3 - 6 cm was used (factor for density conversion = 0.9 and for production = 0.857). Densities of testate amoebae were estimated by direct microscopy of stained (aniline blue MERCK) preparations. Before counting, the stained soil solution was prefiltered trough a nylon net (10 µm mesh size) to remove small clay particles, which otherwise would have masked the tests. Naked amoebae and zooflagellates were enumerated by a most probable number method using ten- and two-fold dilutions in

tissue culture plates. The protozoa were cultured in soil extract medium and inoculated with Enterobacter aerogenes as food source.

Production of testacea was calculated according to Lousier (1974) using the decay data of Meisterfeld & Heisterbaum (contribution in this volume). Unlike the original method, only statistically significant differences between numbers of empty tests were used in estimating the fraction of amoebae produced but which had only been represented by shells. The production of naked amoebae and flagellates were calculated as simple increase of abundance.

Biomass of the species of testate amoebae was estimated by first calculating the volume of the cell assuming an ellipsoidal shape and a specific gravity of about 1.0 (Heal 1970). These data were converted to dry weight by using a factor of 0.2. For naked amoebae and flagellates the cell volume of representative morphological types were calculated by using linear measurements of microphotographs. In relation to the frequency of the different shape and size classes a weighted average was calculated and converted to dry weight. For all sampling dates 0.323 g d.wt. (amoebae) and 0.02 g d.wt. (flagellates) per 10^9 individuals were used. These values are only one-third of those of Clarholm (1981).

To calculate the consumption of protozoa the data of Rogerson (1981) were used. For a temperature of 10 ^{0}C, which is near the annual mean of the site studied, he gives gross production efficiencies for Amoeba proteus in the range of 4 - 29%, depending on the amount of food available. In our calculations we used a value of 20%.

RESULTS

Testacea

The total densities of active testacea in the 0-3 cm layer fluctuated relatively little. Minimal abundances of 17 x 10^6 were observed in July while maximum densities were reached in June. Cysts, which could be observed at most

sampling dates, never reached more than 30 % of the active amoebae. For certain sprecies, e.g. small Euglypha sp., there is some evidence of an inverse relationship between cyst numbers and active forms. Highest biomass figures (1.6 g w.wt.) were found during April, due to the dominance of larger species like Centropyxis aomorpha, Cyclopyxis kahli and Schwabia terricola. The pattern of empty test fluctuations is significantly different from that of the active testacea. There are at least three peaks with maximum numbers of 319 x 10^6 tests in May, June and August. These changes give a first hint of the underlying production, which is only incompletely reflected by the fluctuations of the living amoebae.

Table 1. Mean densities and biomass of relevant groups of soil organisms in the 0-6 cm layer.

	N x m^{-2}	mg x m^{-2} (d.wt.)
Naked Amoebae	3.5×10^9	1133
Testate Amoebae	84×10^6	343
Flagellates	2.7×10^9	54
Protozoa (total)		1530
Nematodes 1)	3.5×10^5	1075
Enchytraeidae 2)	13.7×10^3	473
Lumbricidae 3)	155	9800

1) Heitkamp & Meisterfeld (1982), 2) Mellin (1982), 3) Schaefer (1982)

During the interval between April and October 908 x 10^6 per m^2 discrete individuals and 19.0 g m^{-2} biomass (w.wt.) were produced in the top three centimeters. Maximum production reached 84 x 10^6 individuals and 2.2 g biomass (w.wt) per m^2 and week. On the basis of these data annual production in the 0 - 6 cm layer can be calculated as 3.58 x 10^9 individuals and 14.72 g (d.wt.) per m^2 (Tab. 2).

Naked amoeba and flagellates

The population dynamics of naked amoebae and flagellates is characterized by rapid changes of densities. Abundances in the 0 - 3 cm layer fluctuate more distinctly than in 3 - 6 cm depth. Minimum amoebal numbers are 75×10^6 m^{-2} (0-3 cm) while a maximum of 11×10^9 was observed. Densities of naked amoebae and zooflagellates can increase five- to ten-fold within one week. But in most cases growth is slower. Periods of biomass-buildup last normally 1 to 3 weeks. The mean population size in 0 - 3 cm and 3 - 6 cm are similar although densities per g soil decrease with depth. Annual production of naked amoebae (Tab. 2) amounts to 45.5×10^9 individuals m^{-2} (0 - 6 cm) and for flagellates 48.9×10^9. This corresponds to a biomass (d.wt.) of 14.68 g and 0.978 g respectively. Biomass turnover (P/B) is for naked amoebae lower (13) than for flagellates (18).

Table 2. Annual production, biomass turnover and consumption of Protozoa in a beech forest.

	Production		P/B	Consumption
	$N \times 10^9$ m^{-2}	g m^{-2} (d.wt.)		g m^{-2} (d.wt.)
Amoebae				
0-3 cm	24.5	7.91	13.3	
3-6 cm	21.0	6.77	12.6	
	45.5	14.68		73.40
Testacea				
0-6 cm	3.59	14.72	42.9	73.60
Flagellates				
0-3 cm	27.1	0.542	17.3	
3-6 cm	21.8	0.436	18.4	
	48.9	0.978		4.89
Protozoa (total)		30.378		151.89

DISCUSSION

On a biomass basis there is a large dominance of naked amoebae (74.1%) followed by testate amoebae (22.4%) in the soil under study. Due to their small size, flagellates contribute only (3.5%) to the protozoan standing crop although their densities are similar to those of the amoebae. The quantitative impact of the different protozoan taxa is reflected by their production. Naked amoebae and testate amoebae have similar annual secondary production (14.7 g m^{-2} d.wt.). These values were calculated in two different ways, however. While for testacea the factors of mortality and test disappearance were incorporated, for naked amoebae and flagellates only biomass increase could be used, which probably underestimates true production. These methodological differences explain in part the different biomass turnover (P/B), which is for testacea three times larger than for the other protozoan groups.

The numbers of naked amoebae are in agreement with those reported by Clarholm (1981). Annual production estimates for amoebae as well as for flagellates in forest soils are not available. For testaceans some comparisons can be made between our results and the data of Foissner & Adam (1981), Lousier & Parkinson (1984) and Schönborn (1982). In their study of Testacea in an aspen woodland soil Lousier & Parkinson found a three times higher annual mean density (261 x 10^6 m^{-2}) but a lower mean biomass (0.723 g m^{-2} w.wt.-0.144 g d.wt.). The annual biomass production was 41.2 g (d.wt.) and 2.8 times higher than in the beech forest near Göttingen. The biomass turnover (285) is by a factor of 6.3 faster as our P/B estimates (42.9). Schönborn (1982) in his study of a mull humus in an ash-maple forest found nearly identical P/B values (44), the mean biomass and production, however are lower than in the present study because only the 0.5 cm deep humus layer was investigated. Foissner & Adam (1981) estimated in high mountain soils of the central Alps an annual biomass turnover of 5 in their most productive site.

Comparing the standing crop of protozoa with those of other groups of soil animals Tab. 1. it is obvious, that in terms of biomass the lumbricidae are the most important group followed by protozoa and nematoda. If it is correct that P/B is inversely related to the body size of the species (Petrusewicz & Macfadyen 1970) than the small protozoa should contribute even more to the carbon flow in this ecosystem.

The protozoa need to produce 30.4 g biomass 151.9 g microbial biomass (d.wt.). Mean standing crop of the microorganisms is estimated as 240 g m^{-2}, the bacteria contributing 30 - 60% to the total microbial respiration (M. Vanselow pers. com.). Soil protozoa are main consumers of microbial biomass. While flagellates feed on bacteria and only to a lesser degree on dissolved organic substrates, most small gymnamoebina and testate amoebae are grazer of bacteria, fungal spores and yeasts. Whether larger naked amoebae and testacea are able to use to a significant amount fungal mycelia is unclear. Even if the dimensions of the food particles lie in a range that allows ingestion by phagotrophic protozoa, the quality of the food organisms plays a central role. Many microbial species are inedible (Heal & Felton, 1970) because they are toxic or produce toxic exudates. Therefore, only an unknown fraction of the total microbial biomass is usable for protozoa. Selective grazing pressure on edible microorganisms should change the composition of the microfloral community. Protozoa annually consume 63.3% of the mean microbial biomass. Of this however only 40 - 60% are assimilated. A large part of the ingested biomass is egested unused. Assuming that 26% of the ingested food is respired, then the estimated respiration losses should be approximately 10% of the annual tree and herb litter input of 410 g d.wt..

If these assumptions are correct, then protozoa play a substantial role in the carbon flow of this beech forest ecosystem.

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COMPARATIVE MICROFAUNISTIC STUDIES OF VARIOUS ALKALINE SOIL ASSOCIATIONS IN HORTOBÁGY NATIONAL PARK (HNP)

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The alkaline soils differ from other soil types not only in their physical and chemical characteristics, in their special and extreme properties, but also in their biology. Generally, studies of the microflora of alkaline soils are neglected and our knowledge is rather limited.

According to Bokor /1933/ and Vojnowa-Vodenitscharow /1956/, the microbiological activity of these soils is low. Szabó et al. /1959/ hold that the bulk of the microflora is comprised of species of Streptomyces.

Varga /1956, 1960/ found that Hungarian alkaline soils have an impoverished microfauna.

The purpose of our studies was to investigate the composition of the microfauna /protozoa/ in association with various plants, the seasonal dynamics and to detect the ecological demand of the species.

MATERIALS AND METHODS

The sampling area is at the border of Nyirőlapos and Nyárijárás and belongs to the HNP territories. This region is under strict control for the preservation of its nature. It contains various types of solonetz soils /Arany 1926,1956, Szabolcs 1954/.

In higher regions there are medium crusty meadow solonetz soils while in deeper regions there are degraded solodised solonetz soils. In the deepest sites, to which humus is washed by rain the crusty solonetz is in the process of

changing into meadow solonetz and this is often in contact with the shallow water which gathers in the low sites.

The gradual decrease in the thickness of the A layer and the appearance of the B layer at the surface is made more evident by the mosaic-like associations of plants /Soó 1933, 1980/. The sampling sites were within these mosaics.

EXAMINED ASSOCIATIONS OF PLANTS

I.	Achilleo-Festucetum pseudovinae /A/	/Soó 1933/
II.	Artemisio-Festucetum pseudovinae	/Soó 1933/
III.	Camphorosmetum annuae	/Soó 1933/
IV.	Pholiuro-Plantaginetum tenuiflorae	/Wendebg. 1943/
V.	Artemisio-Festucetum pseudovinae /B/	/Soó 1933/
VI.	Agrosti-Beckmannietum	/Soó 1933/
VII.	Puccinellietum limosae	/Soó 1930/

Samples were taken from two depths 0 to 5 cm and 10 to 15 cm for each plant association.

Quantitative and qualitative analyses of the microfauna were carried out according to the dilution methods used by Singh /1955/, Stout /1967/, Bamforth /1973/, Bick /1972/ and Buitkamp /1977/. For accuracy, it was found appropriate to use a 1:5 dilution with 5 g soil and 25 ml water. Fifteen duplicated cultures were incubated in Petri dishes with non-nutrient agar. The results were analysed using Fisher and Yates' /1948/ statistical presence-absence method. In order to determine the number of cysts, active individuals were first destroyed by incubation at 60°C for one hour.

RESULTS AND DISCUSSION

The changes in the total number of individuals of the microfauna, the humus content and in the total count of viable bacteria are shown in Fig.1. It is evident that there is close correlation between the humus content and the total count of viable bacteria. Although the total numbers of bacterial and various abiotic factors such as the water content temperature and the physical state of the soil.

The distribution of the microfauna in relation to the

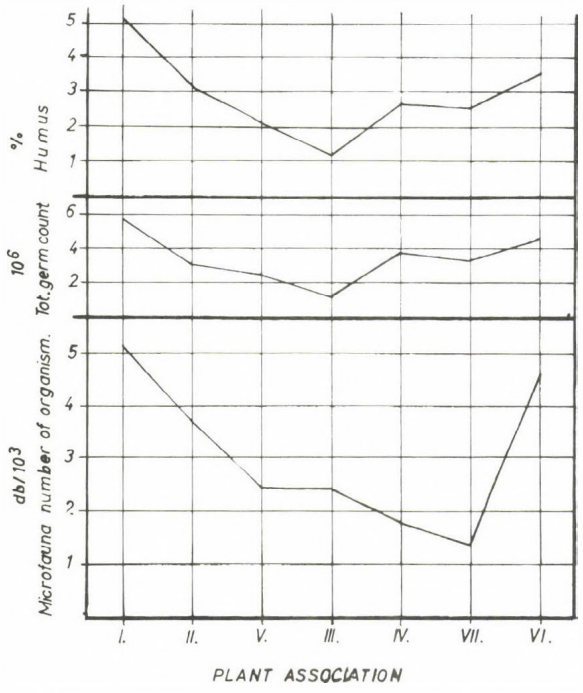

Fig.1. Changes in the total germ count and in the numbers of organisms in plant associations.

plant associations is shown in Fig.2. Flagellates make up the bulk of the microfauna in all samples. The maximum numbers were encountered in the summer /July/ in the upper layers of the A-Fp /A/ associations /14.000 individuals per gram with 70% of them active/. In May and in the dry period from September to October the numbers decreased significantly. Even then, the active organisms amounted to 80 to 100% of the total.

Associations between flagellates and plants can be only illustrated quantitatively.

The distribution of rhizopods was greatly influenced by the water content of the soil. They were encountered in the soils in early spring during wet weather or after rainfalls in the summer. When the humidity of the air dropped below 15 to 20 sp% then the amoebae became inactive.

Of the groups of protozoa studied, the ciliates were examined in greatest detail. We encountered 15 species. The

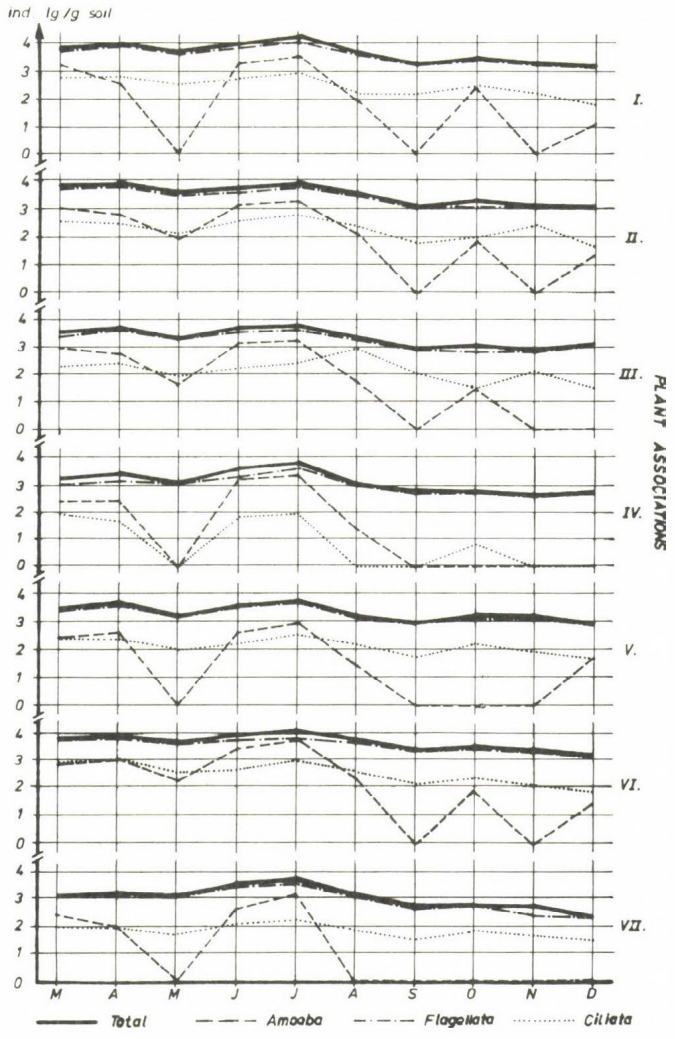

Fig.2. Quantitative changes in the protozoon fauna in the plant associations under survey.

number of individuals and of species appears to be highest in the A-Fp, the Art-Fp and the A-B associations. This is the result of the high water content and well-aerated nature of these soils consequent upon the large pore size, and as a result of the presence of bacteria, amoebae and flagellates which may serve as food. The species most usually encountered were Colpoda cucullus, Colpoda inflata, Euplotes charon, Paruroleptus piscis and Uroleptus halseyi.

Although ciliates were present in summer when humidity was low, their numbers were depressed to 50 to 80%. These places rapidly warm up or become dry and present conditions which are unfavourable for ciliates. Under these conditions they may move deeper into the soils, to regions with higher water content.

If we consider the numbers of species and of individuals we can see that the greatest abundance is encountered in the A-Fp-A, A-Pp-B, p-Pt, Pl and Ca sites.

The dry weather of the early autumn led to a general decline in the numbers of organisms. This was followed by a damp period but without an associated increase in numbers. This may result from physico-ecological factors or from damage to cysts /Gellért 1957/.

SUMMARY

In summary the microfauna is abundant in places with a rich vegetation and with a high humus content. In regard to plant associations, highest numbers are found in associations with A-Fp, A-Fp-A and A-B. In these sites the most common ciliates were Colpoda inflata, Paruroleptus piscis and Uroleptus halseyi. The occurrence of ciliates appears to depend on free scope, on oxygen supply and on the presence of nutrients. The numbers appear to be most influenced by the water content of the soil which needs to be at least 50 sp% and by the nutrient content.

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ADVANCES IN VETERINARIAN PARASITIC PROTOZOA

I. FAUNISTICS AND EPIDEMIOLOGY

Symposia Biologica Hungarica 33 (1986)

COMMENTS ON MYXOSPOREAN LIFE CYCLES

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The mode of transmission of myxosporeans is still quite unsufficiently known and experimental infections mostly fail. That is why Wolf and Markiw (1984) could present a surprising but by other authors not yet confirmed report on transmission of Myxobolus cerebralis involving a tubificid as an intermediate host and also comprising another different life cycle within that host under the disguise of an actinomyxidian, Triactinomyxon.

The sequence of life cycle stages within the fish host and the morphogenesis and structure of these stages, however, are in the same need of clarification as the mode of transmission itself. Recent observations on some previously unknown developmental sequences and on myxosporean ultrastructure have considerably enlarged and complicated the existing knowledge on myxosporean life cycles. That is why we feel it necessary to comment on some of the points.

1. Enveloped condition. This is a term which we propose for the phenomenon that myxosporean cells harbour inner cells inside them, one enveloping the other. As a rule, the inner cells (= secondary cells) arise within the outer ones (= primary cells) by endogeny. This pattern can be followed throughout the whole life cycle.

There is still no experimental data on how exactly do the generative cells originate within the plasmodium as it develops from the sporoplasm. Observations on the origin of the third degree of the inner cells, of the tertiary cells, within the bloodstream developmental stages of Sphaerospora renicola (called "Csaba stages" or "UBO stages", for "unidentified blood organisms") clearly suggest that this happens by endogenous cleavage. At this time, one of the products of the nuclear division becomes what we may call the vegetative nucleus while the other daughter

nucleus within its own new cytoplasmic district is enveloped by two membranes. The membranes originate from the endoplasmic reticulum. The inner membrane turns into the cell membrane of the new generative cell and the other forms the wall of the vacuole within which the generative (or secondary) cell is confined.

Within their primary cells, all secondary cells are accommodated in such closely fitting "vacuoles", sometimes as a group within a single vacuole. The primary cells may represent large multinucleate plasmodium (Myxobolus) or small uninucleate sporogonic pseudoplasmodia (Sphaerospora). The secondary cells are before all the generative cells in large plasmodia and the sporogonic cells in pseudoplasmodia. In the "UBO stages" the secondary cells may harbour inside them, also within a vacuole, tertiary cells. In Hoferellus, a strange myxosporean organism from the kidney of carp to be discussed later, there are even quaternary cells.

The only non-enveloped stage in the myxosporean life cycle is the sporoplasm, except, however, for Kudoa lunata (and possibly other kudoas?) where one sporoplasm cell is situated within the other (Lom et al., 1983).

Both the nucleus of the primary cells (i.e., vegetative nuclei in the plasmodia) and the secondary cells (i.e., generative cells in the plasmodia) may continue division further on.

The origin of the sporoblast cells within the pansporoblast envelope cells is different, of course. Sporoblast cells are not a product of the endogeny of the envelope cells but are the progeny of the sporogonic cell previously encircled and enveloped by the pericyte.

The enveloped condition has the only true parallel in marteilid haplosporeans (Marteilia,Paramarteilia) and its intricacy is only exceeded by Paramyxa paradoxa where the innermost cell of the spore is a quinquenary cell. This similarity between myxosporeans and marteiliids is obviously just a convergence. The enveloped condition is as characteristic of Myxozoa as is the presence of polar capsules.

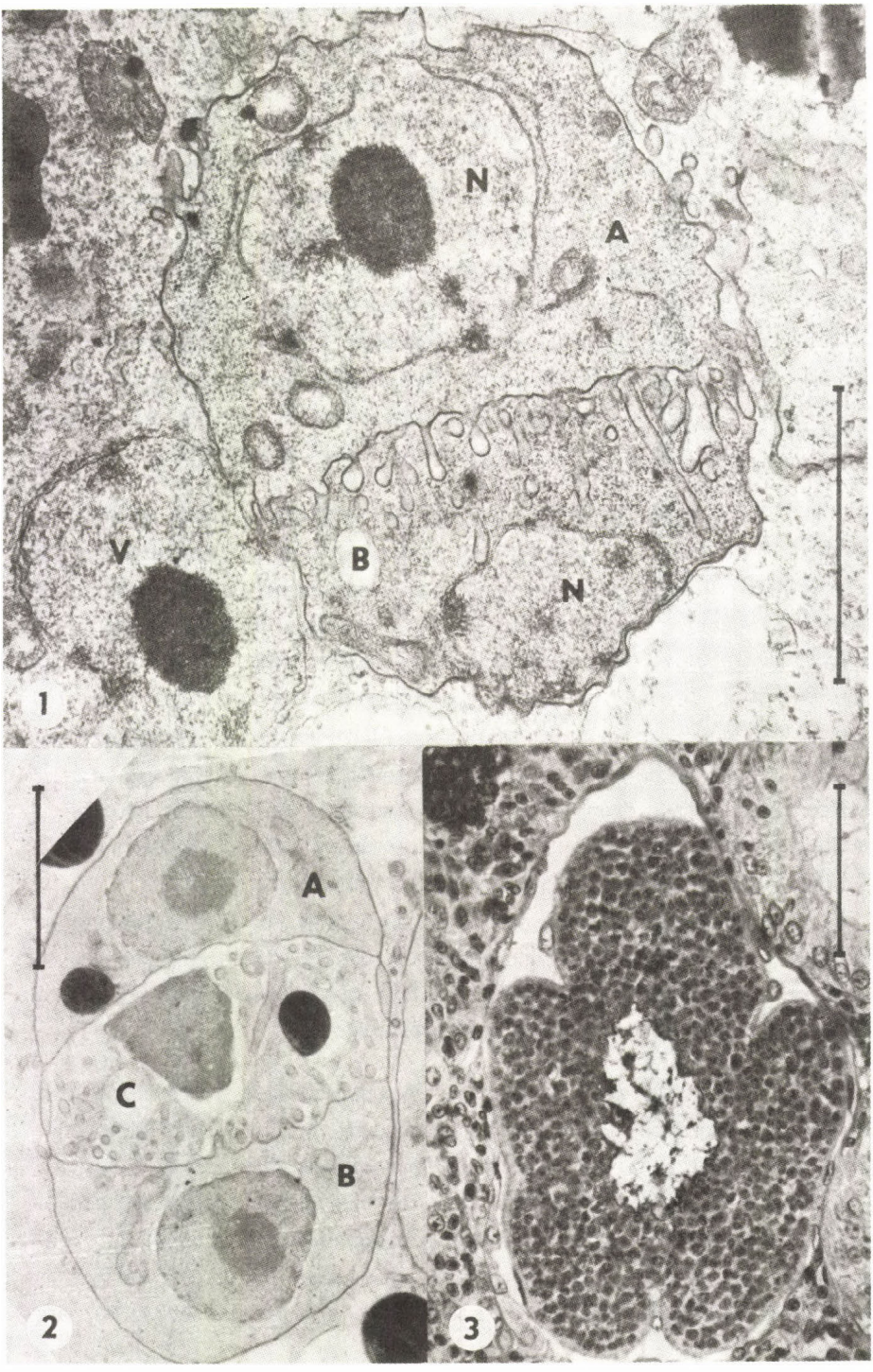
2. Pansporoblast formation. The union of pericyte and sporogonic cell giving rise to a pansporoblast is typical of species with large plasmodia such as Myxobolus, Myxidium or Henneguya. In species with small pseudoplasmodia such as Sphaerospora or Ceratomyxa there is no pansporoblast formation and the spores are simply produced by proliferation of the sporogonic cells within the pseudoplasmodium, the latter being sometimes considered homologous - not quite correctly - with the pansporoblast.

The absence (e.g., in Sphaerospora) or presence (e.g., in Mitraspora) of pansporoblast formation may be a differentiating character of at least some genera. In the genus Kudoa some species form small pseudoplasmodia while other (K. lunata) may form large plasmodia which produce spores without pansporoblasts, simply as a group of differentiating and interacting sporogonic cells. Thus the "large" and "small" kudoas may have the same mode of sporogenesis. Factors controlling polysporic vs. mono- or disporic state in a given genus or species also elude our understanding. Thus trophozoites of Myxidium lieberkühni in a single population in the urinary tract of pike produce large plasmodia in the urinary bladder and small ones both in the bladder and in the renal tubules. Large plasmodia are polysporic with the formation of pansporoblasts, and the small stages often produce two spores only (also within a pansporoblast ?).

3. Behaviour of generative cells. Grassé and Lavette (1978) described in plasmodia of Sphaeromyxa a special kind of generative cells, the lobocytes. They were supposed to have a scavenger function and to be capable of ingesting sporogonic cells. In plasmodia of various species of the genus Myxidium we have found a curious way of union of two to three cells. One cell pierces by numerous pseudopodia the other one (Fig. 1) or two cells "sandwich" a third one (Fig. 2) piercing its cytoplasm from two sides. The pierced cells seem to be bound to perish. The active, piercing cells differ from the lobocytes and this union of cells differs from the union of pericyte and sporogonic cell. Is this a special case of feeding of generative cells by predation on their kins ?

In M. rhodei several pericytes each with its already encircled sporogonic cell may stay in close contact, adhering together across a thin layer of plasmodium cytoplasm. In other genera such early stages of pansporoblast formation stay always separately.

4. Extrasporogonic stages of myxosporean life cycle belong to developmental sequences which do not produce directly spores and which probably serve for proliferation of the parasite within its fish host. They are morphologically defferent from the sporogonic stages and occupy other sites. Although they are not the prerogative of the genus Sphaerospora, they are best known in species of this genus as the "Csaba" or "UBO" stages in the bloodstream (Csaba, 1976; Lom et al., 1983; Molnár,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1984) and as the swimbladder stages (Csaba et al.,1984; Körting et al., 1984) of Sphaerospora renicola. Sporogonic stages of this species are found in the renal tubules of the common carp.

Bloodstream parasitism, so curiously displayed by the "UBO" stages is manifested by various myxosporean organisms. In Myxobolus encephalicus, the immobile polysporic plasmodium is fixed in the brain blood vessels of carp. In Sphaerospora molnari, developing pseudoplasmodia and sporoblasts may circulate freely in the blood, and so do the developmental stages of S. ohlmacheri from frog tadpoles (Desser and Lom, unpublished), while their final sites of infection are the gills and the kidney, respectively. The "UBO" bloodstream stages are thus far known from five cyprinid and one centrarchid fish species, each of them infected with a different Sphaerospora species. The swimbladder Sphaerospora stages were recorded from tench, too (Prof.Körting, personal communication).

We still ignore the precise sequence of these cycles; although they probably precede spore formation, they may - notably the "UBO" - persist for a long time after the sporogonic stages have developed in the renal tubules.

A further important example of extrasporogonic stages are the "PKX" cells associated with the proliferative kidney disease (PKD) of rainbow trouts and other salmonids and considered to be the causative agent thereof. They are found in the kidney interstitium, sometimes in the bloodstream. Ultimately, they reach the lumen of the renal tubules where they transform into sporogonic stages (Kent and Hendrick, 1985). They have the characteristics of the myxosporean cells (e.g., microtubular bundles at the nuclei of inner cells) and resemble the bloodstream

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Fig. 1. Two generative cells in the plasmodium of Myxidium lieberkühni; one (A) pierces with its finger-like cell projections the cytoplasm of the other (B). N - their nuclei; V - vegetative nucleus of the plasmodi- μm. Bar = 4 μm. - Fig. 2. A complex of two generative cells in the plasmodium of a Myxidium sp. from the gall bladder of Cottus bairdi. Pseudopodia of (A) and (B) pierce the cytoplasm of (C). Bar = 4 μm. Fig. 3. Nephrocystidium pickii: a growing"xenoma" with a hypertrophic nucleus and numerous myxosporean cells in the cytoplasm. Bar = 50 μm.

stages of S. renicola in comprising, within the primary cell, secondary and tertiary cells. A unique feature are the electron dense inclusions within the primary PKX cells. They have been formerly mistaken for haplosporosomes from which they differ in that their inner core is thimble shaped. We have to remember that dense inclusions, sometimes reminiscent of haplosporosomes, are found in sporoplasms of most myxosporean species.

In the axons in the brain of a minnow, Notropis cornutus, carrying a heavy infection with a Myxobolus sp. in the muscles and the brain, we have found small plasmodia with numerous cells obviously of myxosporean nature (Ferguson et al., 1985). We have recorded axonal sheaths distended with similar organisms in brains of carps from localities infested with Myxobolus encephalicus. Both cases may constitute another case of extrasporogonic stages; we can speculate on their astonishing way of getting into and out of the axonal cylinder. Similar stages were recorded by Marquet and Sobel (1970) in the axons of a lungfish, Polypterus enlicheri and earlier by Stensas et al.,(1967) in the axons of Bufo arenarius - amphibians are also known as hosts of myxosporea. Such stages may not be as unique as it might appear.

5. Intracellular development. As a rule, myxosporeans were considered to be parasites of body cavities and of intercellular spaces, which definition does not encompass multivalvulid genera developing within the myocytes and some early reports on some other myxosporeans. Myxobolus cyprini develops within the myocytes of carp and several other cyprinids (Molnár and Kovács-Gayer, 1985 ; Dyková and Lom, unpublished) and several myxoboli were recently discovered in myocytes of North American cyprinids (Desser and Lom, unpublished).

Mitraspora cyprini and Sphaerospora renicola also have intracellular stages in the renal tubule epithelium (Ahmed, 1973; Dyková and Lom, 1982). Myxidium lieberkühni has intracellular stages in the renal tubules (Dr.D. Bucke,personal communication; Lom and Dyková, unpublished) which consist of secondary cells within primary ones. Of other examples, we shall quote just two cases, Hoferellus cyprini and Nephrocystidium pickii. These organisms are of special interest since they also induce a remarkable hypertrophy in the infected cell.

The organisms identified as H. cyprini invade epithelial cells of the renal tubules of carp which become hypertrophic. Massive proliferation of the parasites ultimately destroys the normal structure of the

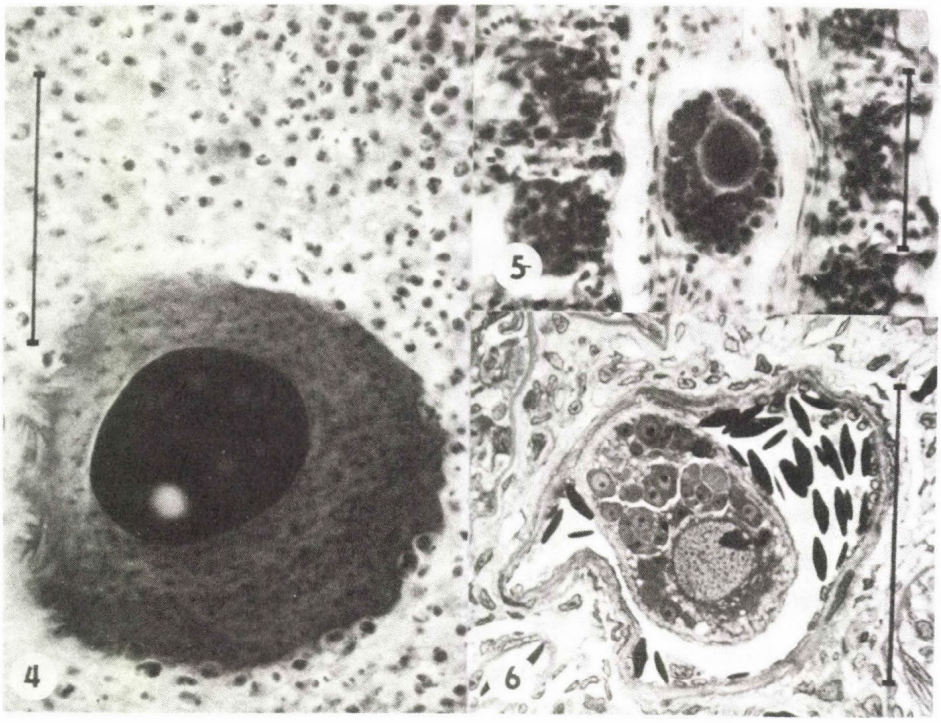

Fig. 4. Nephrocystidium pickii : a detail of the hypertrophic nucleus of the host cell with an appearance different from that in Fig. 3, with a large nucleolus and myxosporean stages in the cytoplasm. Bar = 50 μm.
Figs 5 and 6. Early stages of plasmodium development of Thelohanellus pyriformis. 5 - cytoplasm filled with generative cells includes a hypertrophic host cell attached by a thin stalk to the endothelium of the blood vessel. Bar = 50 μm. - 6 - a semithin section of a plasmodium attached to the endothelium. Note the early sporoblasts and the large nucleus of the hypertrophic host cell. Bar = 50 μm.

epithelial cells and turns the tubular wall into an enormous cyst-like structure packed full with the parasite. No sporogenesis could ever be found. The stages ultimately degenerate. We consider them to belong to the life cycle of Sphaerospora renicola as its developmental blind alley (Dyková et al., 1983). "Hoferellus" is not a phenomenon unique for carp. We have seen similar "Hoferellus"-like organisms in the kidney of Leuciscus cephalus and another one was photographed by Yokote (1982) in the kidney of Plecoglossus altivelis.

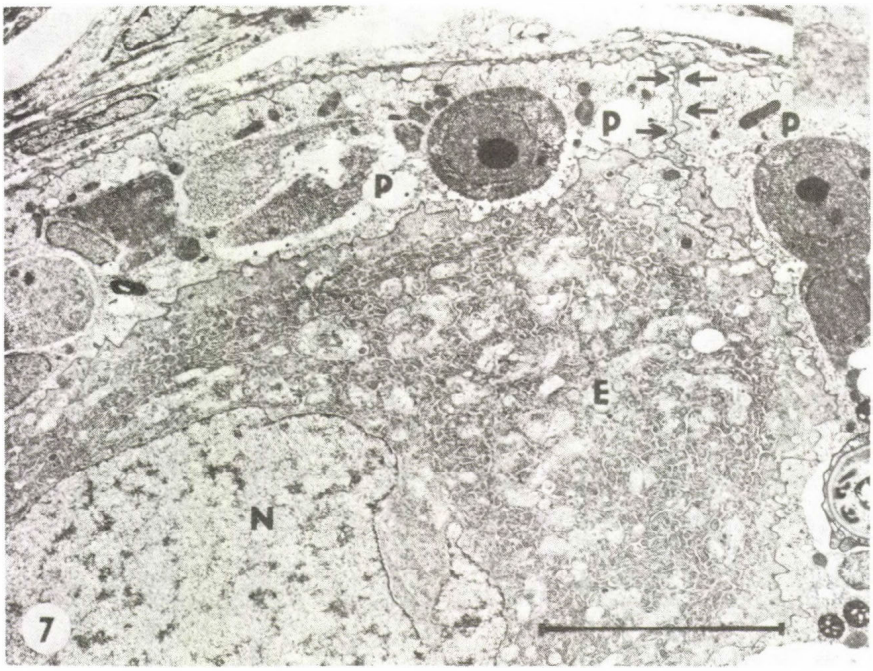

Fig. 7. Thelohanellus pyriformis. Part of the hypertrophic host cell (E) inside the plasmodium and its nucleus (N). A narrow cytoplasmic strand (arrows) between the encircling parts of the plasmodium (P) connects it with the endothelium. Bar = 10 μm.

A still more striking example of hypertrophy due to intracellular myxosporeans is offered by Nephrocystidium pickii, described as an enigmatic myxosporean by Weissenberg in 1921. It infects endothelial cells of glomerular capillaries and turns them into huge xenoma-like structures similar to xenomas provoked by microsporidians of the genus Glugea. The hypertrophic cells have the size up to 0.5 mm with a huge central nucleus (Fig. 3). The cytoplasm is repleted by small primary cells (4 - 6 μm) each containing two secondary cells (Fig. 4). The cell structure (bundles of microtubules close to the nucleus, lack of centrioles etc.) is typically myxosporean. No sporogony has ever been observed. There is an evidence that all the parasite cells in such a xenoma are ultimately degraded and eventually destroyed by the host tissue reaction. We presume that Nephrocystidium might be in fact a developmental blind alley in the

life cycle of Myxidium lieberkühni : all pikes infected with the former also carry an infection with the latter.

We have observed a curious case of what may be called "contact" hypertrophy in Thelohanellus pyriformis. Plasmodia of this species develop within the branchial blood vessels of tench (Tinca tinca) being attached to the endothelial cells (Figs 5, 6). One of these cells becomes hypertrophic (Fig. 7) and is enclosed within the plasmodium which does not, however, pervade its cytoplasm. Possibly, the enlarged host cell with proliferating endoplasmic reticulum and an enormous area of contact with the parasite serves for a better nutrition of the parasite.

The examples of intracellular parasitism by Nephrocystidium and Hoferellus offer two more conclusions. While the intracellular stages of Kudoa and Myxobolus do not harm the myocyte beyond the area where it is actually being digested, infection of other types of cells may induce their hypertrophy and, eventually, a gradual and complete destruction. Further, the abortive, aberrant development in myxosporea may not only affect some spores in the process of formation but it may involve whole developmental sequences. They end up neither in sporogony nor in transfer of the infection to other regions of the host body or to another host.

The developmental cycle of myxosporeans as we have known it until recently can be compared to the tip of an iceberg - many of its stages have been virtually unknown and only recently have begun to surface. One conclusion that can be reached is that myxosporean life cycle may not follow an identical pattern in all of their groups, neither in the mode of spore formation, nor in the character of extrasporogonic stages, nor in the sexual phenomena for which there is just a scant circumstantial evidence and, possibly, not even in the way of their transmission.

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PATHOGEN EFFECTS OF AMOEBAE ON FISH AND MAMMALS: PRELIMINARY RESULTS

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INTRODUCTION

Amoebae of the genera Acanthamoeba and Naegleria have been implicated as the etiological agents of amoebic meningoencephalitis in man and animals. Many studies are concerned with pathogen effects in man, but little is known about the host distribution and the potential pathogenicity of amoebae in fish. Large fish-kills of trout (Sawyer et al.1978) occurred in hatcheries. The cause of these fish-kills was attributed to amoebae isolated from peritoneal fluid, intestinal mucosa and gills. After isolation, experimental infection of amoebae was tried in freshwater fish by Taylor (1977) and Franke and Mackiewicz (1982).

Numerous strains of free living amoebae have been isolated by one of us from ground water and rivers in Alsace (France) and from organs of wild trout caught in the same rivers (Derr-Harf and Monteil, 1983). This study is concerned with pathogen effects of reference strains and strains of amoebae isolated from Alsatian rivers.

A comparison is made between pathogen effects of amoebae strains inoculated in vitro on cell cultures and in vivo in mice and trout.

MATERIAL AND METHODS

Isolation and culture methods

Water samples were collected in rivers in sterile 50 ml containers, centrifuged and inoculated on non-nutrient agar spread with Enterobacter

aerogenes (NNE) for isolation of amoebae. Acanthamoeba and Naegleria strains were cultured axenically in SCGYEM as described by De Jonckheere (1977), and incubated at 28 or 37°C.

Pathogenicity tests

Amoebae were tested for cytopathic effect (CPE) on C_6 rat glial cells, EPC Epithelioma papulosum cyprini cells from carp Cyprinus carpio, RTG_2 rainbow trout gonad fibroblasts and BF_2 bluegill fibroblasts. One ml from an axenic culture containing 10^5 amoebae counted in a haemocytometer was added to the cell culture directly after the last medium change. Rat glial cells were incubated at 28 or 37°C, fish cells at 20°, both with 5 % CO_2, and were examined daily for CPE up to 7 days, using the following pathogenicity scale :

CPE 1 : small areas of destruction in cell culture
CPE 2 : widespread areas of destruction
CPE 3 : isolated islands of cells left
CPE 4 : complete destruction of cell monolayer

Amoebae from the same axenic cultures were tested for virulence in mice and trout. Three-weeks old mice were anesthetized with ether and 0.03 ml of the culture was inoculated intracerebrally. Mice were observed for symptoms of meningoencephalitis for three weeks. Portions from the brain were inoculated on NNE for isolation of amoebae. Small rainbow trouts (Salmo gairdneri R.) weighing 8 to 25 g were anesthetized with MS 222 (100 mg/l) and inoculated with axenic culture as follows:

- 0.05 ml intramuscularly anterior to the dorsal fin
- 0.2 ml intraperitoneally
- 0.03 ml in the nasal cavity closed by a Histoacryl (Braun) layer
- 0.015 ml intracerebrally.

Water samples from aquaria were examined for amoebae. Pretest and control trout were autopsied. Test trout were observed for symptoms for two months and autopsied. Samples from brain, kidney, spleen, muscle, gills and intestinal content were plated on NNE for isolation of amoebae.

Strains

The following strains have been tested :

Strain		Origin
2005b	Acanthamoeba sp.	Lachter river, Derr-Harf
Lilly	Acanthamoeba culbertsoni	Molet
PP397	Naegleria australiensis ATCC 30958	De Jonckheere
KUL	Naegleria fowleri ATCC 30808	De Jonckheere

RESULTS

The preliminary results reported in this study indicate that each amoeba strain tested induced the same cytopathic effects on the three fish cell lines, EPC, RTG_2 and BF_2 (Table 1).

Table 1 : Cytopathic effects of amoebae on rat and fish cell cultures : Hours to destroy the cell monolayers inocubated with 10^5 amoebae

Strain	C_6 rat glial cells		Fish cell lines (20°C)		
			EPC	RTG_2	BF_2
2005b	48 H	28°C	48 H	ND	48 H
N.australiensis	24 H	37°C	24 H	24 H	24 H
N.fowleri	96 H	37°C	168 H	ND	ND
A.culbertsoni	48 H	37°C	168 H	168 H	168 H

ND = Not Done

Cytopathic effects differed in fish and mammal cell lines with A.culbertsoni and N.fowleri. The two strains are virulent for humans and mice. But they had a rather slow effect on C_6 rat glial cells and a very slow effect on fish cell lines. N.australiensis achieved complete destruction of the monolayer in 24 hours, the river strain 2005 b in 48 hours.

Pretest and control trout were free of amoebae. Some water samples from the aquaria were contamined with free-living amoebae from different genera than those inoculated.

Intramuscular, intraperitoneal and intranasal inoculations of strain 2005b had no effect in trout and no amoebae were isolated from samples of brain, kidney, spleen and gills. Small amoebae, of a different genus were isolated from the intestinal content of one trout. Nine days after intracerebral inoculation with N.australiensis and the river strain 2005b, some trout were ill-balanced, but there was no gross pathology or mortality. The strains were isolated from the brain of ill trout. N.fowleri and A.culbertsoni were not isolated from the brain of test trout. In mice, N.australiensis, N.fowleri and A.culbertsoni induce mortal meningoencephalitis within 3 to 8 days, but strain 2005b has no effect.

Table 2 : Virulence of amoebae after intracerebral inoculation in mice and trouts

Strains	Mice	Trout
2005b	-	+
N.australiensis	+	+
N.fowleri	+	-
A.culbertsoni	+	-

DISCUSSION

These preliminary results show a good correlation between pathogen effects in trout and cytopathic effects on fish cell lines. This correlation does not exist between mice and rat cells (Derr-Harf and De Jonckheere, 1984). The incubation temperature does not seem to interfere with cytopathic effect, as N.australiensis takes 24 hours for CPE4 on C_6 rat glial cells at 37°C, as well as on fish cell lines at 20°C. Cytopathic effects in vitro and virulence in vivo differ in fish and mammals with several strains of amoebae. It seems from this study that mammals and fish show a different sensitivity to amoebae. As it happens

for bacteria and viruses the virulence of amoebae might be different in mammals than in fish. Further studies should be made on routes of possible inoculation in aquatic environment and host-parasite relationship in fish.

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HOST SPECIFICITY IN THE COCCIDIA OF SMALL MAMMALS: FACT OR FICTION?

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Today I hope to stimulate some thought about how we may better understand host specificity of the intestinal coccidians (sensu strictu: Eimeria, Isospora spp.) found in natural populations of small mammals. Host specificity, even in such a restricted sense, requires that many things interact, in the proper sequence, to insure the successful completion of a coccidian life cycle. This complex host-parasite relationship can be distilled into 4 major components: (1) The Oocyst, its identification [22], and all factors that result in transmission; (2) the total milieu of The Host, including its age, sex, nutritional state, genetic constitution, immune status, occupation by other parasites and microbes, behavior, feeding habits, etc.; (3) The Ecosystem, into which the unsporulated oocysts are deposited including the biotic and abiotic factors that contribute to each oocyst's sporulation and transmission to a new host or to its demise; and (4) The Coevolutionary Process that unites the first 3 factors and from which they cannot be separated. Thus, the brief discussion of each component that follows naturally involves overlap with 1 or more of the other components.

THE OOCYST

The oocyst is the most readily available stage in the coccidian life cycle. Although many descriptions are inadequate, and some host records unreliable, the literature on the structure of sporulated oocysts is nonetheless the most abundant source of information available about which species of coccidia infect small mammals. It is from these resources that we first began to make inferences about host specificity in the coccidia despite probable inadequacies in measures of specificity [sensu 15, p.174].

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In mammalian Eimeria and Isospora spp., as far as we know, the unsporulated oocyst is almost always passed with the host feces. Numerous biotic and abiotic factors then work in concert and, given enough oxygen, moisture and warmth the oocyst will sporulate, sometimes in a matter of hours. The sporulated oocyst then must wait until a "suitable" host ingests it before development can continue. Using quantitative and qualitative characteristics of sporulated oocysts, Levine [17] estimated there can be at least 2,654,736 different species in the genus Eimeria alone, probably an underestimation. Clearly, there is no shortage of features that can help us distinguish among oocysts of different species, yet oftentimes oocysts are found in the feces of 1 host taxon (A) that are identical to those found in another host taxon (B). If A and B are congeners we accept the coccidium as a valid parasite of both. If, however, host B is less related to host A, we are taught to conclude that this is a spurious finding of oocysts from host A which are just passing through the gut of host B or to designate the parasite as a new species despite its striking similarity to the species described from host A. We do this for at least 2 reasons. First, dogma tells us that eimerians, and to a lesser extent isosporans, are highly host specific, being limited naturally to a narrow range of host species [23], generally within 1 host genus [16]. Second, we are unwilling to accept that host A oocysts found in host B represent a real parasite of host B because of the negative results of cross transmission studies [16,18,19, 23]. Although some of these studies were done carefully, most did not give such pertinent information as the origin, strain, or age (and therefore viability) of the oocysts used as inocula or the immune status or genetic strain of their recipient hosts. Many times such information is not available. Nonetheless, as we will see, these and other factors all contribute to the probability of oocysts successfully completing their development in a host--a cornerstone of the concept of host specificity. I am not trying to discredit over 150 reports of negative attempts to cross transmit eimerians between various mammalian hosts (mainly rodents [18] and herbivores [19]), but I am saying that these, as well as the dozen or so attempts that were positive, should be viewed in a broader perspective.

Within the last 16 years, a number of published cross transmission reports lend credibility to the notion that some mammalian coccidia are flexible in their host requirement. Todd and Hammond [41,42] showed that 2 eimerians, E. callospermophili and E. larimerensis, were naturally found in, and could be experimentally cross transmitted between, ground squirrels

(Spermophilus spp.) and a prairie dog (Cynomys sp.). De Vos [5] demonstrated that E. chinchillae, originally isolated from the chinchilla, could be experimentally transmitted to 7 genera of wild rodents (2 families) and to laboratory rats and mice. Mayberry and her colleagues [26,28] showed that successful transmission of a rat coccidium, E. separata, was possible in certain genetic strains of laboratory mice. In our work at the University of New Mexico we have been surveying various groups of small mammals from large geographic areas (Canada, Japan, Mexico, South America, USA). As we measure and identify oocysts of hundreds of coccidia species from thousands of host specimens, we often see nearly identical oocysts from geographically syntopic host species whose genetic relationships are highly variable. For example, we have seen oocysts of E. tamiasciuri, first described from the red squirrel, Tamiasciuris hudsonicus in Arizona (USA) [20], in 7 species of squirrels (Sciuriidae) representing 3 genera (Eutamias, Sciurus, Tamiasciuris) and in an unrelated host, Microtus montanus (Arvicolidae). Similarly, we have found eimerians and/or isosporans to be shared between different genera of shrew moles (Dymecodon, Urotrichus), moles (Mogera, Talpa), and cricetid rodents (Reithrodontomys, Peromyscus, Neotoma, Baiomys) [33,34,44, unpublished data]. In all cases, hosts that shared coccidian species were distributed syntopically. The coccidia are generally thought to share a long evolutionary history with their hosts (association by descent). Ewald [13], suggested that after a parasite had penetrated the original defenses of a long-exposed population of the same host species, then the parasite should be able to overcome defenses of a new, but similar, unexposed host species. Mammals as a group tend to be biochemically similar and it is reasonable to assume that several genera of syntopic hosts that have similar nutritional requirements, would provide intestinal milieus similar to one another. Thus, the possibility exists that among syntopic host species, each with its own long-established coccidia fauna, and each living in the same general environment for thousands of years, a particular genetic or ecologic situation occurred which permitted transfer of a coccidium to a new host species (association by colonization). Once a new host is colonized, selection may operate on the parasite population within that host, resulting in strains better able to infect other members of the "new" host species. Selection for the capacity to reproduce in the new host species, while simultaneously maintaining the ability to reproduce in the first host species, may thus expand the parasite's range and reduce host specificity. The concept of host transfer and

the mechanisms that lead to it are discussed in detail by Mitter and Brooks [30]. The fact that a hundred or so cross transmission experiments cannot repeat such events does not necessarily mean that under natural conditions where millions of random cross transmission events take place, that such successful host transfers could not occur.

Obviously we have more to learn about host and parasite genetics before the question of host specificity can be answered conclusively in these instances, but the circumstantial evidence suggests that perhaps some coccidia are more euryxenous in natural environments than we believe and that geographic and ecologic factors play more important roles in host specificity than previously believed, as first suggested by Pellerdy [31, p.43].

THE HOST

When a sporulated oocyst is ingested by a host, the interaction begins a complex series of events and processes. To begin infection, excystation is mandatory or nothing else will happen. For the coccidia, this does not present an insurmountable problem because nearly all studies have shown that excystation of sporozoites is a rather nonspecific phenomenon [23]. Once sporozoites are exposed to the gut milieu, is it the properties of the luminal contents or of the gut cells that determine the first process of infection? In a naive, susceptible host, sporozoites seem to be able to penetrate and leave enterocytes with apparent impunity. Once within a host cell, the mechanism by which a zoite completes its development, or not, is unknown. Trager [43] stressed that intracellular protozoan parasites somehow induce the host cell to assist actively in their nutrition. Marquardt et al. [24] summarized the evidence on nucleolar hypertrophy in host cells with coccidian developmental stages and suggested that protein synthesis (via transcription) in specific host cells was critical for a specific coccidium to complete its development. Basically, their argument stated that the coccidia share at least a portion of their genome with their host and that this relationship is so exquisitely fine-tuned that when a zoite enters a host cell and signals for transcription to begin, a gut cell in the "right" host will respond whereas a similar cell, in the "wrong" host, will not respond. Nucleolar enlargement, however, is not a universal response of cells infected with coccidia. Additionally, a coccidium that is a generalist in its host requirements could presumably satisfy its needs by "turning on" the same or similar genes of gut cells in several related host taxa. This could produce nucleolar hypertrophy without strict host specificity. A related hypothesis regards nuclear enlargement in infected cells

as due to an increase in DNA, an attempt by the host cell to maintain its nucleo-cytoplasmic ratio (i.e., a non-specific response in which the host cell perceives the parasite as additional cytoplasm) [21]. This argument is no more universally supported than Marquardt's given the variability in host cell response to the presence of coccidians.

Completion of an infection by eimerians usually confers upon the host some degree of specific resistance to reinfection; thus, resistance in a host population tends to be directly correlated with age. Prior infection with coccidia results in immunity that is primarily cell-mediated [35] although antibodies may play some part, especially in modifying primary infections [38]. For example, Rose et al. [38] found IgA to E. neischulzi in bile and intestinal washings of rats after both primary and challenge infections. The inference from these and other workers [3,7,36] is that some of the effects of immunity, whether innate or acquired, are exerted on the zoite before penetration of enterocytes. Presumably, IgA attaches to the zoite surface and inhibits/delays its penetration of enterocytes. Those zoites that do penetrate enterocytes are thought to be sufficiently altered by digestive enzymes in the gut lumen to be unable to develop properly.

We know that strains within each host species are not uniformly susceptible/resistant to particular parasites [25,28,37] and Shirley [39] showed that there was (sometimes considerable) variation in pathogenicity, reproductive index, immunogenicity, and enzyme electromobility among 5 strains of E. necatrix. It follows that host and parasite genotypes must play a major role in susceptibility/resistance to coccidial infections, and this may explain some of the negative results of published cross transmission studies (i.e., the lack of life cycle completion by 1 strain of coccidium in 1 strain of host is not sufficient proof that other strains within the host species cannot serve as hosts).

Host nutritional state, competition for space/nutrients by concurrent infection with other parasites, and the resultant cross-immunity (or other effects) also must be taken into account when looking at host specificity. For example, people with iron deficiency anemia induced by hookworms are particularly resistant to bacterial infections. Only this type of anemia confers such protection, for people with other types of anemia are fully susceptable to bacterial infections [4]. As most hosts in nature are known to be infected with more than 1 type of parasite, similar phenomena may be

discovered that contribute to what we superficially view as host specificity in certain host-parasite assemblages. Such topics have not received much attention by parasitologists.

We assume the association between vertebrate hosts and their coccidia must be an ancient one because coccidia have been found in almost every vertebrate species that has been examined for them. Most host species are known to carry at least 1 coccidian species, many hosts have several, and under natural conditions, some portion of a host population (1-100%) will be found to be actively discharging oocysts. Hosts examined by my students, colleagues and me over the years can be placed into 1 of 3 categories:

(I) Those with a high prevalence of infection and most infected individuals harboring 2 or more coccidians. Hosts in this category include rabbits (Sylvilagus), pikas (Ochotona) and moles (Condylura, Scalopus, Talpa). Of 331 hosts sampled, 325 (98%, range 96-100%) were infected at the time of sampling and 275 of these infected hosts (85%, range 83-87%) had 2-6 eimerians concurrently [8,11, unpublished data].

(II) Those with a low prevalence of infection and most infected hosts harboring only 1 coccidium. Most hosts fall into this category, including bats (Tomopeas), deermice (Peromyscus), ground and tree squirrels (Eutamias, Sciurus, Tamiasciurus), jumping mice (Zapus), kangaroo rats (Dipodomys), kit foxes (Vulpes), shrews (Blarina, Sorex), voles (Microtus), woodmice (Apodemus) and woodrats (Neotoma). Of 1785 hosts examined, only 592 (33%, range 8-54%) were infected at the time of sampling, but 527 of these infected hosts (89%, range 77-100%) had only single species infections by coccidia [10,12,27,33,34,40,44, unpublished data].

(III) Those with intermediate values for both infection rate and single/multi-species infections. Only the shrew-moles (Dymecodon, Neurotrichus, Urotrichus), so far, fall into this intermediate spot between the 2 larger groups. Of 69 hosts sampled, 56 (81%, range 71-100%) were infected at the time of sampling, but only 24 of the infected hosts (43%, range 17-56%) had single-species infections when collected [9, unpublished data].

Unfortunately, most authors do not present their survey data in a format that allows one to determine if hosts infected with coccidia can be placed into one of these categories. Nonetheless, the question must be asked, "Why is the 1 host-1 coccidium association (Cat. II) so dominant in naturally-occurring host communities, especially when most host species are known to serve as good hosts for 2 or more coccidia?" The reasons for the

observed infection patterns are obscure, but some insight may be gained from the ecological and evolutionary history of the hosts.

THE ECOSYSTEM

Each of the factors already discussed or alluded to plays a part in determining host susceptibility/resistance and such factors are often greatly affected by environmental considerations. For example, Cat. I hosts are all ancient lineages of mammals, tend to be morphologically and chromosomally more conservative and show less heterozygosity than most other groups, and have not undergone the extensive radiation seen in many other mammalian orders. They are also either fossorial, semi-fossorial or, at least, associated with burrows or crevices [1]. Such habitats provide oocysts with stable, moist environments and can act as concentrating mechanisms, thus making viable oocysts readily available to the hosts in these habitats. This, in part, may contribute to the high infection rates seen in Cat. I hosts. If we consider also the potential for increased survival of these oocysts in the external environment, we might predict that the coccidia species in Cat. I hosts each need to produce fewer oocysts to reach new hosts than do those coccidia species in Cat. II hosts. Because of the ancient nature of Cat. I hosts, it is likely their coccidia have been associated with them for a long time and this could lead to a high degree of phylogenetic relatedness among these parasite species. Thus, hosts that always have many coccidia (Cat. I) may have species that are more closely related to each other than are those in hosts (Cat. II) that usually have only 1 coccidian at any point in time. How such potential relationships may contribute to host specificity as we measure it is unknown.

Cat. II hosts tend to be surface-active species (feeding, breeding, etc.), generally have undergone extensive adaptive radiation, and many are characterized by higher levels of genetic variability. Although many of these hosts inhabit holes, they are thought to defecate elsewhere. Oocysts exposed to surface environments (drying, UV radiation, etc.) are less likely to be readily available to vagile hosts that traverse such places and lower levels of host infection may be expected. As highly specific parasites may be at risk of extinction in areas where transmission is precarious, selection may favor the ability of these coccidia to colonize new hosts. To accomplish this, coccidia that live in Cat. II hosts should require many more oocysts be produced than do their congeners in Cat. I

hosts and such increased burden on the host can correlate with increased pathogenicity/immunogenicity. Ewald [13] predicted that a primary pressure toward increased severity by parasites is competition between genetically distant parasites and this may be partially responsible for the trends we see in Cat. II hosts (i.e., their coccidia are competing against potential colonizers from other host species).

In certain habitats, transfer of parasites to other hosts that live in that habitat may be facilitated by ecologic factors [2]. The possibility that syntopic hosts have a similar surface such as feathers in the case of mites or the mucous membrane of the gut in coccidial infections may be examples of such factors [29]. The question we face is simply which component of the observed distribution of parasites derives from phylogenetic factors and which component derives from ecologic factors? Analyzing the parasite-host-environment relationship is difficult because the ecosystem is complex and the relationship is in a steady state condition which is easily disturbed by experimental intervention. For example, Doran [6] examined 6 species (11 subspecies) of kangaroo rats (Dipodomys spp.) for coccidia in California. Only D. panamintinus mohavensis (251 specimens examined) was found infected with E. mohavensis whereas none of the other host subspecies (360 specimens), all living syntopically with D. p. mohavensis, were ever found to be infected. However, in his cross infection studies, all 10 other host subspecies were susceptible to infection with E. mohavensis and several proved to be "better" hosts (based on oocysts discharged). It appears that some factor (host behavior or a microenvironmental isolating mechanism) kept the majority of susceptible hosts living in the region from encountering the parasite under natural conditions. Therefore, even though a similar ecology strongly favors transfer to other conspecific or congeneric hosts, as has been demonstrated in many host-parasite systems [29], failure to find infection with coccidians in a potential host, where it appears to have ample opportunity to become infected, does not mean that the host is not suitable.

THE COEVOLUTIONARY PROCESS

The coevolution of host taxa and their respective parasite faunas must certainly be a driving force in the susceptibility/resistance of hosts to parasites (i.e., host specificity). Most systematists have long assumed that hosts and their parasites have radiated in parallel and have focused mainly on the question of whether the parasites in different hosts could be

used as indicators of host or parasite phylogenies [14]. Obviously the picture is much more complex than that and we do not clearly understand the ecological forces constantly interacting with genetic and other factors that enable parasites to utilize either a broad or a narrow spectrum of hosts.

What if many host species are not available in certain environments (e.g., tundra)? Host specificity may result then from the absence of host animals rather than from strong selection pressures for specialization on 1 or a narrow range of hosts [32]. In other words, one would expect the degree of host specificity to increase as distance from the center of diversity of the major host taxon increases. Does this apply to the coccidia? Would the specificity of these coccidia be "released" with an influx of new hosts?

Whether or not we are discussing host specificity, the concept of coevolution calls for our attention as parasitologists because it allows for a synthesis of ecology and evolution (and therefore genetics) of both the hosts and their parasites [14]. Historically, parasitologists have considered each species (host or parasite) as an entity isolated from associated species (of hosts or parasites) and the environment, which has been assumed to remain constant, has been largely ignored. Obviously the biological world of our parasites is much too complex to allow us to continue along this path if we are to begin to truly understand it.

CONCLUSIONS

Are the coccidia of small mammals in natural populations highly host specific? I suppose in some instances (e.g., tundra) where hosts are few and far between, they probably are. In most mammalian populations, however, I think they are somewhat more flexible in their host requirement, especially when there are plenty of hosts in the same general environment that may be within their familial phylogeny and/or that may become exposed due to their ecological habits. Coccidia cannot survive without their hosts and this puts strong pressure on them to adapt to their environment and to the hosts available within that environment. As Holmes [15, p.185] has suggested for helminths, I believe the coccidians can exhibit "rapid evolutionary change and track local conditions." And the local conditions are much more complex than most of us like to remember.

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A SURVEY OF OVINE COCCIDIOSIS

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INTRODUCTION

Recently attention has been directed to the intensive rearing of lambs in Hungary. Since Kotlán's examination (1929) we have practically no information on the rate of coccidial infection and on the occurrence of Eimeria species present in sheep in this country. The aim of this survey was to assess the infection rate in lambs and to determine the occurring Eimeria species.

MATERIALS and METHODS

Seventeen flocks of lambs in different parts of Hungary were examined for the presence of coccidial oocysts. Faecal samples were collected randomly from the rectum of twenty individuals in each flock. In two additional flocks the naturally acquired coccidial infection was followed up over several weeks. In Gomba 20 lambs were marked and faecal samples were taken fortnightly six times in the autumn and five times in the spring. In Demecser samples were taken from 40 lambs on two occasions before weaning. At the time of weaning the lambs were divided into two groups. One group was kept indoors and fed a pelleted diet. The other group grazed outdoors and received a daily feed supplement of 650 gr. Samples were obtained from 10 to 15 animals of each group on each occasion. On the last day further 90 specimens were examined from the lambs kept indoors. Oocyst counts were made on a total of 754 samples using the McMaster-slide. Specimens with oocyst per gram (OPG) higher than 100 were placed for sporulation in 2 % $K_2 Cr_2 O_7$ at 27°C. After

six days the oocysts were differentiated into species according to Levine and Ivens (1970).

RESULTS

All the 294 specimens originating from 15 different sources were examined. The age of lambs in weeks, the rate of infection, the mean OPG and standard deviation (S.D.) obtained for the groups are shown in Table 1. In the majority of flocks the oocyst counts showed considerable individual variation from animal to animal. The maximum OPG count found in the faeces of a lamb was 1 890 000. The results of examinations in Gomba are summarized in Table 2. Lambs were excreting oocysts at three weeks of age. Oocyst production reached the peak at 5 to 6 weeks of age at the time of weaning in both periods studied. In autumn a second peak was detected in 9 week old lambs.

Table 1. The rate of infection and faecal oocyst counts in a total of 294 lambs reared in 15 different areas of Hungary

No. of flocks	Date	Age (weeks)	Rate of infection	OPG (mean ± S.D.)
1.	Nov., 1982	5	17/20	125 417 ± 441 768
2.	May, 1983	6-7	20/20	14 880 ± 25 721
3.	May, 1983	4-6	20/20	6 170 ± 10 903
4.	May, 1983	4-6	15/15	7 187 ± 6 460
5.	July, 1983	10	19/20	725 ± 1 255
6.	July, 1983	8	20/20	8 885 ± 7 993
7.	Sept.,1983	8-12	20/20	2 362 ± 2 540
8.	Nov., 1983	5	24/25	43 645 ± 56 468
9.	Nov., 1983	12	12/17	167 ± 217
10.	Nov., 1983	4-8	9/20	135 ± 193
11.	Nov., 1983	7	15/20	1 810 ± 3 700
12.	Dec., 1983	7-8	19/20	960 ± 1 036
13.	Dec., 1983	5-7	20/20	2 210 ± 2 493
14.	Febr.,1984	6-8	14/15	10 523 ± 20 253
15.	Febr.,1984	3-5	15/22	9 372 ± 23 095

Table 2. The rate of infection and mean oocyst counts (±S.D.) in faeces of 20 lambs examined during two seasons (Gomba)

Date	Age (weeks)	Rate of infection	OPG (mean ± S.D.)
1982			
Sept. 23.	3-4	11/20	9 662 ± 23 719
Oct. 8.	5-6	20/20	242 371 ± 531 928
Oct. 20.	7-8	20/20	7 357 ± 8 688
Nov. 3.	9-10	20/20	26 490 ± 69 824
Nov. 7.	11-12	20/20	5 845 ± 5 820
Dec. 2.	13-14	19/20	947 ± 1 329
1983			
Marc. 3.	5-6	20/20	27 765 ± 101 187
Marc.17.	7-8	15/20	26 719 ± 114 388
Marc.31.	9-10	10/20	156 ± 394
Apr. 14.	11-12	7/20	30 ± 56
Apr. 28.	13-14	3/20	77 ± 257

Table 3. The rate of infection and mean oocyst counts (± S.D.) in a total of 240 faecal samples (Demecser)

Group of lambs x	Date	Age (weeks)	Rate of infection	OPG (mean ± S.D.)
1983				
A	June 20.	4-5	39/40	9 610 ± 24 213
A	July 6.	6-7	40/40	4 160 ± 5 215
B	Aug. 9.	11-12	10/10	1 705 ± 2 206
C	Aug. 9.	11-12	10/10	1 380 ± 1 669
B	Sept. 14.	16-17	10/10	4 560 ± 9 367
C	Sept. 14.	16-17	10/10	4 200 ± 10 848
B	Oct. 5.	19-20	14/15	993 ± 1 350
C	Oct. 5.	19-20	15/15	493 ± 276
B	Oct. 5.	19-20	87/90	1 960 ± 6 289

x A - unweaned lambs
B - lambs kept indoors
C - lambs kept outdoors

It was followed by a reduction in the number of oocysts. The output from individuals varied from 0 to 1 720 000 during this examination. Results of the survey conducted in Demecser are shown in Table 3. The mean oocyst count obtained for the group kept indoors surpassed that of the other group at all the three times studied. Outstanding OPG values were found in the faeces of 4 to 5 weeks old lambs. Variability in oocyst production was high within individual lambs of both groups resulting in the high S.D. Characteristically high S.D. occurred not only in the groups of 10 to 15 animals but in a group consisting of 90 lambs. Despite the large number of oocysts produced none of the lambs displayed clinical signs of coccidiosis. Throughout this experiment mixed infections were present. The incidence of 11 species of Eimeria are listed in Table 4.

Table 4. Incidence of species of Eimeria in 570 faecal samples

Species	Incidence (%)
Eimeria ovinoidalis	51.5
Eimeria ovina	37.7
Eimeria parva	24.0
Eimeria faurei	20.2
Eimeria ahsata	16.4
Eimeria crandallis	8.6
Eimeria intricata	4.0
Eimeria marsica	3.3
Eimeria pallida	1.3
Eimeria granulosa	1.2
Eimeria punctata	1.1

DISCUSSION

The newborn lambs may contract the infection from the environment contaminated by their dams immediately after birth. The shortest praepatent period is 11 days as recorded by McDougald (1979). Therefore it is not surprising that as young as three weeks old lambs pass oocysts. In the present survey

oocyst excretion increased up to the age of 4 to 6 weeks, and decreased thereafter. Similar observations were made by Pout et al. (1966). The second peak obtained in the flock of Gomba could have been due to an impared resistance occurring around the time of weaning. Apparently punctate oocysts were observed on eight occasions always in the form of mixed infection in agreement with the work of Landers (1955). However, doubts have emerged in regard to the identity of E. punctata since certain authors considered it as an abnormal oocyst of E. ovina Joyner et al. (1966). The diagnosis of clinical coccidiosis has been proven very difficult and a correct diagnosis may be achieved by using the formula of Gregory et al. (1980). High individual OPG counts were found on some occasions and E. ovinoidalis, a species thought to be pathogenic, was present in 51.5 % of lambs but no clinical symptoms were observed.We can conclude that coccidial infections are potentially present but clinical disease rarely occurs in Hungary.

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A FIELD TRIAL WITH SOME COCCIDIOSTATS IN A LARGE-SCALE RABBIT FARM

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INTRODUCTION

The range of preparations to prevent intestinal and hepatic coccididosis in rabbits is rather limited comparing to that of poultry. Only a few preparations proved to be effective in the past decades in connection with the above mentioned diseases of rabbit. Dürr and Lämmler /1970/ tried some sulphonamides to prevent intestinal coccidiosis in rabbits. From the four preparations Rofenon /sulphadimetoxine--diaveridine 3:1/ in 100 mg/kg dose proved to be fairly effective against single high dose and mixed oocysts infection. Fitzgerald /1972/ found Monensin effective in 0.005 and 0.02 % concentration to prevent hepatic coccidiosis. According to Lämmler and Hein /1980/, Salinomycin in 20 and 45 mg/kg dose is effective against E.stiedia infection. Sambeth and Raether /1980/ examined the anticoccidial effect of ionophore antibiotics /Salinomycin, Monensin, Lasalocid/ in rabbits in case of preventive dosage. Salinomycin an Monensin in 50 mg/kg dose prevented the development of intestinal and hepatic coccidiosis effectively. Varga /1982/ examined among others the effectiveness of Salinomycin under large--scale conditions. According to his experimental results Salinomycin in 50 mg/kg dose provides good anticoccidial effect.

On the basis of the above, our target was to try Salinomycin, Roferon, Monensin and Furazolidon under large-scale conditions.

MATERIAL AND METHODS

The experiments were carried out under large-scale conditions with 7058 New Zealand white, weaned rabbits at the age of 28-30 days at Agricultural State Farm, Környe. In the course of the feeding experiment repeated three times the rabbit feed of identical composition was supplemented with 25 and 50 mg/kg Salinomycin, 25 and 50 mg/kg Monensin, 100 mg/kg Furazolidon, and 100 mg/kg Roferon. In all the cases an untreated control group was also present. Trials lasted 8 weeks and after a week withdrawal period the rabbits were slaughtered.

The oocyst shedding of the animals was determined by the McMaster oocyst-counting method. Examinations were carried out weekly, using 10-10 mixed faeces at each group.

The effectiveness of the preparations mentioned above was evaluated on the basis of the performance of rabbits /average daily body weight gain, feed convertion ratio/, mortality % and the amount of shedding oocysts /OPG/.

RESULTS AND DISCUSSION

The effect of the tested preparations on meat rabbit production is summarized in table 1., while their effectiveness against coccidiosis is summarized in table 2.

Of the tested preparations only the Furazolidon treatment increased body weight gain significantly /$P < 0.05$/ in the 1st and the 3rd experiment. Neither ionophor antibiotics nor Roferon improved daily body weight gain moreover, in most of cases the body weight gain of the treated animals proved to be worse than that of the untreated ones.

The tested preparations generally moderated the feed intake and increased feed convertion ratio to a small extent. Furazolidon in each case, Rofenon, Salinomycin and Monensin, except the 1st experiment /higher dose/, resulted in better feed convertion ratio than that of control groups.

In most of the cases, except the whole 2nd experiment and the Furazolidon treated group of the 1st experiment, mortality rate in the untreated control group was lower. 92 % of deaths was due to diarrhoea.

Table 1.

The Performance Data of the Trials

Treatment	Body weight gain g/day	Feed consumption g/day	Feed conversion	Mortality %
Experiment 1				
I. Furazolidon /100 mg/kg/	28.72	109.40	3.04	10.6
II. Rofenon /100 mg/kg/	27.70	121.20	3.21	18.8
III. Salinomycin /50 mg/kg/	26.53	113.90	3.34	17.7
IV. Monensin /50 mg/kg/	23.27	116.30	3.50	25.0
V. Control /untreated/	27.66	119.50	3.18	15.5
Experiment 2				
I. Furazolidon /100 mg/kg/	23.88	112.82	3.41	34.34
II. Monensin /25 mg/kg/	23.74	103.47	3.25	28.38
III. Rofenon /100 mg/kg/	24.37	106.93	3.23	28.43
IV. Salinomycin /25 mg/kg/	25.08	113.01	3.36	30.40
V. Control /untreated/	25.67	136.54	3.89	44.68
Experiment 3				
I. Furazolidon /100 mg/kg/	26.13	108.0	3.52	13.88
II. Salinomycin /25 mg/kg/	24.66	87.7	3.16	18.44
III. Monensin /25 mg/kg/	23.83	95.8	3.37	17.14
IV. Control /untreated/	25.10	119.0	3.95	13.61

From the tested preparations Salinomycin and Monensin in 50 mg/kg dose practically eliminated, while in 25 mg/kg dose significantly decreased, the amount of oocysts in the faeces. Furazolidon and Roferon showed no anticoccidial effect in our experiments. However, the effective anticoccidial drugs couldn't decrease losses caused by the diseases of the digestive organs either.

Table 2.

Average oocyst number x 1000 of 1 g faeces in the trials

Treatments	Age in days								
	28	35	42	49	56	63	70	77	84
Experiment 1									
I. Furazolidon /100 mg/kg/	17.6	1.1	6.5	5.5	8.0	1.7	5.8	31,6	2,4
II. Rofenon /100 mg/kg/	13.7	1.1	3,5	21,0	1,7	6,1	18,2	59,6	3.7
III. Salinomycin /50mg/kg/	2.9	0,1	0	0.3	0	0	0,1	0	0
IV. Monensin /50 mg/kg/	3	0,1	0	0	0	0	0	0	0
V. Control /untreated/	16	2	0.8	0.4	0,3	0,3	0,4	0.8	0.5
Experiment 2									
I. Furazolidon /100 mg/kg/	14.2	26.5	14	40	17	3,4	4.4	28.6	3.4
II. Monensin /25 mg/kg/	3,4	1.5	0	0	0,4	0	1.0	0	0
III. Rofenon /100 mg/kg/	3,6	2.3	0	3,5	3,2	2,3	20.7	9,1	11,3
IV. Salinomycin /25 mg/kg/	2,2	0	0	0	0	0	0	0	0,2
V. Control /untreated/	2,5	0.6	0,3	0.4	0,4	0,3	1.5	0.4	0.3
Experiment 3									
I. Furazolidon /100 mg/kg/	1.7	2.2	1.9	13,2	24.2	0,9	43.2	55	
II. Salinomycin /25 mg/kg/	13,4	1.2	1,2	0,2	1.1	0	0.2	0,2	
III. Monensin	1.6	0.6	0.2	0.1	0,1	1,4	0.4	0,2	
IV. Control /untreated/	3	1.9	1.9	2,3	2.5	3,0	6.8	6.3	

The data above are the averages of ten samples.

It is well known that in the etiology of the diseases of rabbit's digestive system, besides coccidiosis, numerous bacteria /E.coli, C.perfringens, B.piliformis /viruses/ adeno-, rota-, reovirus/ and other non-infectious causative agents can be present /10/. Our field trials didn't cover the examination of these agents. The results are likely to suggest that keeping in wire bottom cage under large-scale conditions and regular cleaning and disinfection /often burning/ prevent death due to intestinal and hepatic coccidiosis.

However, Varga /1982/ reports that 54 % of rabbit liver coming from small-scale breeders was condemned because of hepatic coccidiosis at the slaughter house of Agricultural State Farm, Környe. Consequently, mainly small-scale populations need effective coccidiostats. For this purpose 25 mg/kg Salinomycin or Monensin can be suggested.

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TISSUE CYST-FORMING COCCIDIA IN ANTARCTIC VERTEBRATES

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The investigation of parasitic protozoans is one of the actual tasks of biological research in the Antarctic (cf. Odening 1985). Parasitic protozoans have been found as one of the last groups of organisms also in the Antarctic. The first reports of Sarcocystidae (single findings of sarcocysts within the musculature) in penguins and seals date only from the last few years (Ippen et al. 1981, Odening 1983, 1984a, Odening and Zipper, in press).

According to the reviews of Kalyakin and Zasukhin (1975) and Levine and Tadros (1980), there have not been any reports of Sarcocystis from the Antarctic and neither from penguins (Sphenisciformes) or seabirds related to them (Procellariiformes). Obviously no findings of Sarcocystis from other seabirds have been available so far. However, some reports of sarcocysts exist outside the Antarctic in 5 species of seals of the northern hemisphere (Callorhinus ursinus, Zalophus californianus, Phoca vitulina richardsi, Ph. hispida, Erignathus barbatus; cf. Odening 1983) and in 2 species of whales (sei whale, Balaenoptera borealis, and sperm whale, Physeter catodon; cf. Mehlhorn et al. 1976).

The first report of sarcocysts in penguins dealt with a Humboldt penguin (Spheniscus humboldti) from a zoological garden (Henne et al. 1977). The hitherto available, only sporadic Antarctic reports concerned the species Pygoscelis antarctica (chinstrap penguin), P. papua (gentoo penguin), Eudyptes chrysolophus (macaroni penguin) and the leopard seal (Hydrurga leptonyx). All of these Antarctic intermediate hosts

are members of the marine ecosystem (cf. Odening 1984b). Consequently, here a life cycle with an aquatic, in these cases marine phase (sporocysts in the exterior environment), must be presupposed. An aquatic cycle in freshwater, with water-fowl as intermediate hosts, very probably exists at least in the Sarcocystis forms reported from Anatidae (1-3, among them S. rileyi). This does not seem to be sure in the forms from Ardeidae (1), Rallidae (1) and Charadriiformes (5).

It is noteworthy that the overwhelming majority of sarcocyst reports from different bird species (more than 50) point to terrestrial cycles. A similar proportion is found in mammals where essentially only the findings in seals and whales suggest an aquatic cycle (against the reports of infection in about 130 terrestrial species of mammals). Thus the aquatic cycle is relatively rare, and, moreover, the marine cycle is a special ecological case which is connected with more special conditions in the Antarctic. The questions arising from this mainly concern the following topics:

1) Do the sporocysts, shed with the faeces of the definitive host, lie on the bottom of the sea or do they float in the water?

2) What about the viability and longevity of the sporocysts being in the cold Antarctic water?

3) Which are the possibilities for distribution of the sporocysts once in seawater?

4) Which kinds of predator-prey relations come into question for the realization of the life-cycle?

Furthermore, the question arises as to what extent the "classical" thesis of the strong intermediate host specificity of Sarcocystis species is valid. In general there is species specificity in the known terrestrial cycles (with the exception of S. debouei in which possibly only an "order" specificity - Passeriformes - exists; cf. Levine and Tadros 1980, Tadros and Laarman 1982). Interestingly, the species S. rileyi, occurring in freshwater in ducks, seems to have a broader intermediate host specificity (interpreted as "family" specificity by Levine and Tadros 1980). Could this be connected with the aquatic cycle (perhaps with the possibly lesser chance of

ingestion of the sporocysts dispersed in the water by the intermediate hosts)? Also the sarcocysts studied from 2 systematically widely separated groups of whales (toothed whales: sperm whale, and baleen whales: sei whale) did not show essential ultrastructural differences in the cyst wall and could therefore be identical (Mehlhorn et al. 1976). On the other hand, it could be possible that the intermediate host specificity in birds can be broader than in general in mammals. In our Antarctic area of investigation (the South Shetland Islands) 3 species of penguins occur together in some places, partly breeding in mixed rookeries and swimming in mixed groups in the sea in search of food. Here it is necessary to mention that the macaroni penguin does not belong to the 3 species often occurring together in this area; it is only a sporadic visitor there. Our investigations on the morphology of the sarcocysts and cystozoites are not yet finished but point to the presence of a different Sarcocystis species in each of the 2 penguin species (and in the leopard seal). Thus the cystozoites in the chinstrap penguin are elliptical to oval, measuring 2-3 x 1.2-1.4 µm, those in the macaroni penguin are distinctly curved, measuring 7-8 x 1.5-2.2 µm, and those in the leopard seal are slightly curved, measuring 6-7 x 2-2.2 µm.

The corresponding predator-prey relations in the Antarctic can be described as follows. The (adult) penguins are prey of the leopard seal (Hydrurga leptonyx), the killer whale (Orcinus orca) and, perhaps, the southern fur seal (Arctocephalus). The leopard seal is a possible prey of the killer whale. The food relation penguin/leopard seal/killer whale is very stable and guarantees the maintenance of a Sarcocystis cycle (in our area of investigation the relation penguin/leopard seal is permanent in the summer). In comparison with that the relation leopard seal/killer whale does not seem to be so stable. Starting from that we could think of carrion-eating birds as definitive hosts, eventually of giant petrels (Macronectes) or skuas (Catharacta). A cycle effectuated by oral transmission through carrion (of seals, respectively penguins) would be an adaptation to the specific conditions in the Antarctic where carrion remains fresh longer than in warmer regions. The same reflection could

be applied to arctic seals and perhaps also to whales as intermediate hosts. But this is not necessary for an explanation of the main line of transmission from seals, whales and penguins to a definitive host. At present the reports of sarcocysts in carnivores as intermediate hosts are judged sceptically (cf. Tadros and Laarman 1982); it is thought probable that carnivores obtain such an infection with sarcocysts only casually and this would be a dead end. Here we should like to except explicitly the seals (and the whales) from these carnivores, for they do not eat birds or mammals (with few exceptions, the most distinct being the leopard seal and the killer whale). The majority of seals (and whales) are worldwide (and penguins within their area) a normal prey of the killer whale, the seals in the Antarctic (and Subantarctic) in addition are prey of the leopard seal and in the Arctic of the polar bear. Here by all means a mostly stable predator-prey relation exists. This is problematic in the case of the sperm whale which is said to be not hunted by the killer whale. Here - and this simultaneously would be a possibility of application to the terrestrial environment where terrestrial carnivores can be hosts of sarcocysts - the hypothesis of carrion-eating definitive hosts could be a stimulus for further thought.

It should be mentioned finally that unsporulated oocysts were found in the leopard seal close to ruptured sarcocysts (Odening 1984a). The unusual location of the oocysts far from the intestine suggests that they belong to the same species of Sarcocystis found mainly in form of sarcocysts in the leopard seal investigated. By this the "classical" thesis of obligatory diheteroxeny of Sarcocystis would be questioned.

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COCCIDIA OF GAME ANIMALS IN BULGARIA

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Over the last decade, we have carried out investigations of the coccidia of game animals in Bulgaria. Our attention has been given mainly to game animals of economic importance (pheasants, mallard ducks, fallow deer and mouflons, for example) which are raised under conditions of involving partial husbandry. Special attention was given to carnivorous game animals (such as wolves, jackals and foxes) which might act as vectors of sarcocystosis of wild and domestic animals.

During our investigations we have identified 59 coccidian species belonging to 5 genera (Eimeria, Isospora, Tyzzeria, Sarcocystis and Klossia) (see Table 1).

Of the game birds examined, the pheasants were more heavily infected than others which had been bred in farms and subsequently released. The coccidian species which were recovered were E. phasiani, E. colchici, E. duodenalis, E. pacifica and E. megalostoma. The first three of these are of economic importance. The mortality in some farms reaches 30% (Golemansky and Juzev, 1980).

We have studied the incidence of infection of pheasants as a function of their age and found the highest infection (86%) in 40-day-old birds. The rate of infection falls to about 50% in 60-day-old pheasants, and in one-year-old birds has fallen to about 14%. The comparatively low population density of pheasants in nature, their predominantly independent way of life and acquired immunity act as natural barrier against the spread of coccidiosis among natural pheasant populations.

Table 1. Game animals examined and infected with Coccidia in Bulgaria

Hosts	Examined	Number of species				
		Eimeria	Isospora	Tyzzeria	Sarcocystis	Klossia
Aves						
Phasianus colchicus L.	245	5	-	-	-	-
Anas plathyrhynchos L.	314	2	-	1	-	-
Numida meleagris L.	244	2	-	-	1 ?	-
Tetrao urogallus L.	5	2	-	-	-	-
Mammalia						
Bison bonasus L.	16	3	-	-	-	-
Capreolus capreolus L.	159	6	-	-	-	-
Cervus elaphus L.	18	2	-	-	-	-
Ovis musimon Pall.	99	7	-	-	-	-
Sus scrofa L.	91	3	1	-	1	-
Lepus europaeus L.	52	4	-	-	-	-
Sciurus vulgaris L.	27	4	-	-	-	-
Canis lupus L.	3	1	1	-	1	-
Canis aureus L.	32	1	2	-	2	-
Vulpes vulpes L.	146	2	3	-	1	1 ?
Total		44	7	1	6	1

Two species of the genus Eimeria (E. anatis and E. batakhi) and one of the genus Tyzzeria (T. perniciosa) were found in semi-wild ducks. The incidence of infection in ducks was low compared with pheasants, being about 10% in birds 6 to 8 months old.

E. ventriosa and Eimeria sp. were found in blackcocks, and E. numidae and E. grenieri in guinea-hens. Of some interest was the observation of sporulated oocysts in the rectum of two guinea-fowls. These resembled Sarcocystis, but full identification was not possible.

Of the game mammals investigated, the roe deer, hares and mouflons were most heavily infected.

Six species of Eimeria and one species of Isospora were found in roe deer. Arranged in the order of prevalence they were: E. capreoli (30% animals were infected), E. panda (23%), E. ponderosa (10%), E. rotunda (8%), E. catubrina and E. superba (less than 2%). In some cases sporulating oocysts belonging to the genus Isospora were found but these differed in appearance from those of I. capreoli as described by Svanbaev (1959). We consider these findings as transitionally passed oocysts from other hosts, probably birds. The incidence of infection is higher in young hosts (62-75%) than in adults (34-56%).

The mouflons imported into Bulgaria from Hungary and Czechoslovakia have become established as a permanent component of the game fauna. The total infection rate of mouflons is high (79%). Seven species of Coccidia were identified, the following four being the most frequent: E. ovina (67%), E. ninakohljakimovi (40%), E. crandalis (31%) and E. ahsata (21%).

Relatively high levels of infection were also found in hares. The numbers of these animals have shown a downward trend over the last few years. Four species of Eimeria are established: E. semisculpta (88%), E. leporis (72%), E. europaea (48%) and E. hungarica (16%). Liver coccidiosis of hares occurs in sporadic outbreaks but is not of economic significance.

No pathological changes in the digestive system, normally associated with acute coccidian infections, were seen. However, the high incidence of infection of hares by coccidia, and the widespread distribution of these parasites, leads us to suspect

that the coccidia are one of the factors limiting the numbers of these animals in Bulgaria. It is important to note here that our information comes mainly from adult hares which are hunted, but it is with young animals, aged 2-4 months, that there are more considerable losses from coccidiosis.

A total of five species of coccidia from the genera Eimeria, Isospora and Sarcocystis were found in boars. The heaviest and most widespread infections were by E. debliecki (42% host infected), E. scabra (19%) and I. suis (3%). Interestingly enough, we found numerous oocysts and free, mature sporocysts of Sarcocystis sp. in the rectum of a specimen shot in the vicinity of Strandja mountain in August, 1981. It is already known that boars may often be infected with muscular cysts of 2 species of Sarcocystis (Frenkel et al., 1979; Meshkov, 1982). This observation of ripe Sarcocystis oocysts and sporocysts in the digestive system may be taken as further evidence that the boar is a final host of some Sarcocystis species. Most likely, these are parasites of rodents, insectivorous animals or other small mammals.

A total of 7 coccidian species (E. vulpis, E. li, I. vulpina, I. canivelocis, I. vulpis, Sarcocystis sp. and Klossia sp.) were found in 146 fox carcasses examined (Golemansky and Ridzhakov, 1975). A great number of immature and sporulated oocysts of Klossia sp. were found in 2 adult foxes. During the last few years there have been other records of Klossia oocysts in vertebrates (Levine et al., 1955, Golemansky and Jankova, 1973). This suggests that it would be worthwhile to investigate the developmental cycles and host range of Klossia spp. in more detail.

Oocysts and mature sporocysts of Sarcocystis have been found relatively often in foxes, wolves and jackals (10% of foxes were infected and 50% of jackals). This suggests that these animals are important in maintaining sarcocystosis of wild and domestic animals in Bulgaria.

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SARCOCYSTIS-INFECTION IN THREE HUNGARIAN ROE DEER (CAPREOLUS C. CAPREOLUS) POPULATIONS

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INTRODUCTION

Sarcocystis species are very common and widely distributed parasites in the visceral and sceletal musculature of deer, especially in roe deer as reported earlier by Kávai and Sugár (1976), Drost (1977), Dollinger (1981) etc. It is obvious, that the occurrence of the cysts is highest in the oesophagus (gullet)-musculature (Kávai and Sugár, 1976; Entzeroth, 1980). Knowing this and the ease of recognizing cysts in the oesophagus macroscopically we decided to include a study of Sarcocystis infections with other investigations in three roe deer populations in Hungary from 1979. Parallel with it we tried to investigate the relationship between the Sarcocystis-infection and the health status of the host organism.

MATERIALS AND METHODS

In the course of the "Man and Biosphere" UNESCO programme we have investigated roe deer in a forest habitat (population "B" living on a hill-land covered over wood by 90 %), in an intermediate habitat (population "A" situated on the Great Plain of Hungary with 10 % afforestation) and in a field habitat (population "P" not far from "A", but having 1 % wood cover only) in 1979-84. Altogether more than 300 roe deers were involved. In addition to collecting many morphological and ecological data (body- and organ-weights, condition parameters,

status of health, growth-development and reproductive performance) of the roe deer specimens (Sugár et al., 1983) we paid attention to the presence and intensity of some ecto- and endoparasites including Sarcocystis.

The oesophaguses were examined only in well-lit surroundings. The visible Sarcocystis-hoses (cysts) were stick-shaped (1) or the relativly rare ovoid shape (2). These probably correspond to the Type 1 and Type 2 in the study of Entzeroth (1980).

RESULTS AND DISCUSSION

The results of the investigations are demonstrated in Table 1 and Fig. 1. The respective incidences of the stick-shaped Sarcocystis-cysts (Type 1) in the oesophagus of roe deer were evaluated separately by populations and age-groups. The distinction of the six age-groups used to evaluate the roe deer's growth and development helped to clarify the nature of the Sarcocystis infections.

We found no infected animals under 6 months of age (I/1). The infection rate was very low (9.1-14.3 %) in the I/2 age-group and relatively low (27.3-38.9 %) in the I/3 age-group. It rose after the 14th month of age and reached a very high level in all 3 populations (96-96 7 %) after the 26th month of age.

The infective rate of the ovoid form (Type 2) was much lower. It was significant only after the 26th month of age: 26.7 % in "B", 16.7 % in "A" and 30 % in "P" population, respectively. Only one roe of the younger age-groups was infected in visible form.

The subjective intensity of the Sarcocystis-infection (Type 1 only) is also evaluated in Table 1 as low, moderate high and very high. The dynamics of infection demonstrates the influence of the age in the manifestation of the infection.

Table 1.

The extensity and intensity of the Sarcocystis infection in the oesophagus of roe deer

Age group	pop.	N° examined	N° infected n	%	N° by the intensity as low	mod.	high	very h.
adult	B	75	72	96	26	29	14	4
	A	30	29	96.7	16	10	3	-
	P	60	58	96.7	17	32	9	-
II/2+3	B	5	4	80	2	2	-	-
	A	5	4	80	2	2	-	-
	P	16	14	87.5	5	6	3	-
II/1	B	6	2	33.3	1	1	-	-
	A	13	10	76.9	8	2	-	-
	P	4	3	75	2	1	-	-
I/3	B	11	3	27.3	3	-	-	-
	A	27	8	37	8	1	1	-
	P	18	7	38.9	3	4	-	-
I/2	B	15	2	13.3	2	-	-	-
	A	14	2	14.3	2	-	-	-
	P	11	1	9.1	1	-	-	-

B = roe deer population living in forest

A = roe deer population living in an intermed. habitat type

P = field roe deer population; age groups as in Fig 1.

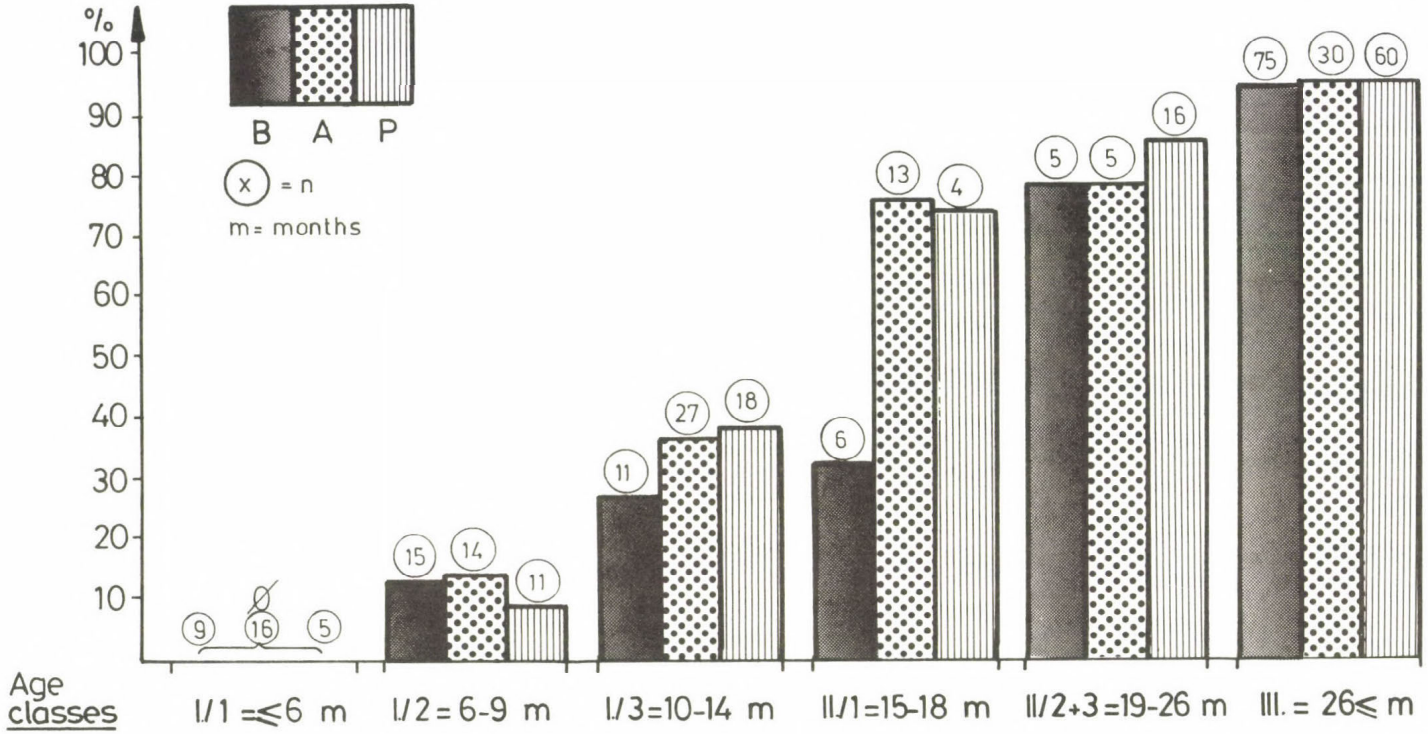

Fig.1. AGE RELATED DIFFERENCES IN THE RESPECTIVE RATES OF SARCOCYSTIS - INFECTION IN 3 POPULATIONS

The uniformly high infection-rate of the 3 populations in the adult age-group illustrates that the biotope-type has little influence on the extensity of the infection.

On the basis of our investigations, the infection and its intensity do not influence the health status or body condition of the animals.

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ENCEPHALITOZOONOSIS IN RABBIT COLONIES

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INTRODUCTION

The microsporidian Encephalitozoon cuniculi (EC), although described by Levaditi, Nicolau and Schoen in 1923, has recetly gained high popularity as 1) the only known microsporidian which occurs frequently in mammals, 2) one of the most common infective agents of laboratory animals, 3) pathogen of economic importance in farms rearing rabbits or polar foxes.

The history of the discovery of the organism, its taxonomic position, host range, pathology, diagnostics, etc. have been recently covered in an excellent review by Canning (1986). The scope of the present paper will therefore be limited to some aspects of rabbit encephalitozoonosis, specifically its diagnostics, occurrence in different types of colonies, transmission under practical conditions of breeding and its influence on performance of rabbits in meat production, as well as to recommended measures for prevention and eradication of infection.

DIAGNOSIS OF RABBIT ENCEPHALITOZOONOSIS

The turning point in the research of EC was the introduction of non-destructive tests for the presence of the parasite. The following methods have been used: indirect immunofluorescent antibody test (IFAT) (Chalupsky et al., 1971), skin hypersensitivity test (Pakes et al., 1972). carbon immuno assay (CIA) (Waller, 1977), complement fixation test (Wosu et al., 1977), immunoperoxidase test (Gannon, 1978), and indirect microagglutination test (Shadduck and Geroulo, 1979).

Of these methods the IFAT has been the most widely used. The second most popular method is the CIA.

IFAT was introduced as the method of detection of EC by Chalupsky et al. in 1971 and was further advanced by Jackson et al. (1973) and by Cox et al. (1972). The advantage of this technique is that it detects the antibodies in both the IgM and the IgG fractions and can, therefore, reveal early infections in which the IgM occurs first (Waller et al., 1978). Another advantage is that it utilizes total antigen which simplifies the procedure and evaluation.

The IFAT reaction is performed according to the protocol given in Chalupsky et al. (1973) but the evaluation of the reaction has undergone some development. Originally, our first experiences suggested the occurrence of a "gap" between negative sera (reacting in titres either 0 or up to 1:8) and the positive sera reacting in titres 1:256 or higher. Further work has, however, shown that sera reacting in the intermediate titres 1:16-1:128 do exist. At present, a positive reaction in serum dilution 1:16 might be considered as the threshold of positivity. The significance of a positive reaction at titres of 1:8 or less remains to be explained as in the case of other diseases diagnosed by IFAT (eg. toxoplasmosis). At present, we consider these reactions as non-specific.

The carbon immunoassay (formerly known as IIR = India Ink Immunoreaction) was introduced into the diagnostics of encephalitozoonosis by Waller (1977), who claimed it to be as sensitive as IFAT. CIA detects only the IgG antibody fraction and so theoretically cannot reveal early infections in which IgM are supposed to occur first (Waller et al., 1978). However, as Lev (1982) has demostrated (by the use of IFAT specifically directed against the IgM and IgG, respectively) the time lapse between the occurrence of these two classes of immunoglobulins in experimentally infected rabbits is very small, being in the range of a few days. Thus the practical disadvantages of the CIA as compared with the IFAT is not very great. According to Lev (1982), in some animals the CIA detectable antibodies appear often sooner than those detectable by IFAT. Recently, the CIA has become a rather widespread method as it provides a more rapid evaluation and does not require specialized equipment except India ink of a proper quality. Some authors now completely rely on this technique.

In our experience, however, the CIA has been less reliable. We have examined 210 rabbit sera and have found a good correlation between CIA and the IFAT in 122 negative and in 76 positive sera. However, 12 sera, which were positive in the IFAT reaction, were negative in the CIA method (Chalupsky et al., 1979b).

THE DISTRIBUTION OF EC IN RABBIT COLONIES

We have investigated the distribution of EC as revealed by the presence of significant titres of antibodies in the IFAT reaction in laboratory colonies, professional farms producing either laboratory or broiler rabbits and in colonies of individual amateur breeders (Chalupsky et al., 1973, 1979a).

Laboratory colonies. The investigation of 3 open laboratory colonies has revealed positive titres in 40% and 29% of animals (Chalupsky et al., 1979a). This confirms the high prevalence of encephalitozoonosis found by several authors in laboratory colonies (e.g. Cox and Pye, 1975: 25-75%; Waller, 1977: 9-82%; Lyngset, 1980: 73%). Crowded conditions in animal rooms, length of stay and the presence of other animals harbouring EC are factors contributing to the high prevalence of the disease in laboratory rabbits.

Professional farms. Despite numerous reports on the occurrence of EC in rabbits, very little is known on the presence of the parasite in farms breeding rabbits on a large scale. Apart from our observations which are discussed below in more detail, the only outhor who has examined commercial farms was Lev (1982), who found 4.7% and 10.2% positive sera in two farms in the FRG.

So far we have examined a large number of sera of rabbits from 7 farms in Czechoslovakia, 8 farms in Hungary and 2 farms in Cuba. Our results are summarized below:

1) EC is not a "necessary evil" contaminating all large rabbit colonies. We have found one farm in Czechoslovakia and 2 farms in Hungary free of infection. However, we cannot explain the absence of the parasite in these colonies and we simply suppose that the parasite was not present in the original breeding stock.

2) On the other hand, some colonies can rate between 20 and 95% of serologically positive rabbits. The exact values are not given here because even at the same farm the number of positive animals can fluctuate depending on the size of the sample and the selection of animals used in the test (breeding does, young rabbits, etc.). At only one farm have we observed clinical signs attributable to encephalitozoonosis (paralysis of hind legs).

Amateur breeders colonies. In our first investigation of the occurrence of EC antibodies in this type of colony we have found no positive animals

in a sample of 60 sera. However, a recent examination of 300 sera obtained from a Czechoslovakian slaughterhouse, which deals with rabbits from private colonies, showed a 6% incidence of positivity (Rubík et al., in preparation). A similar investigation made at a slaughterhouse in Hungary (Vávra et al., unpublished) revealed a 30% positivity.

We think that the difference between our original negative finding and the 6% positivity found more recently can be explained by the present popularity and state support given to the rabbit breeding. This has led to a considerable growth in size of some of the colonies which are now closer in their technology to professional farms than to the old custom of keeping rabbits in small, individually well separated outdoor cages. The increase in size of the colonies may be accompanied by more crowded conditions, less hygiene, more stress etc. At one time, the private breeders in Czechoslovakia could obtain their breeding rabbits from large farms. This practice is certainly responsible for the high prevalence of seropositivity to EC in Hungarian private colonies.

Apart from our data there is very little information on the incidence of encephalitozoonosis in small colonies of amateur breeders. Lev (1982) examined 89 rabbits from such colonies in the FRG and found 5.6% positivity but only in rather low sera dilutions (1 animal reacting in titre 1:80, 1 animal 1:40, 3 animals 1:20). Flatt and Jackson (1970) have examined histopathologically 2,338 rabbits slaughtered for meat in the U.S.A. They have found 4.3% of animals either with parasites or with typical lesions. Unfortunately the authors did not specify the origin of these rabbits.

THE LONG-TERM PERSISTENCE OF EC ANTIBODIES

Our introduction of the IFAT to the diagnosis of rabbit encephalitozoonosis and the finding of high numbers of seropositive animals has prompted us to investigate the long-term dynamics and the persistence of EC antibodies (Chalupsky et al., 1974). In this investigation we examined two groups of rabbits over a two-year period: one group was spontaneously infected and showed high antibody level (minimum 1:526) at the beginning of the examination period and the other consisted of animals originally seronegative which were infected by an intraperitoneal injection of EC spores. In the latter group significant levels of antibodies appeared two weeks after injection and two months later the immune response reached the levels of positivity comparable with that of the first group. Not all animals of

the second group reacted in the same way, but in the course of time all animals reached high levels of antibodies. In both groups high levels of antibodies were maintained (with some fluctuations) for the whole period of the experiment.

Our results were later confirmed by Waller et al.(1978) who found persistent, relatively high antibody levels in a group of experimentally infected rabbits in a 400-day experiment. Bywater and Kellett (1978) also reported a lifetime positivity of infected rabbits with significant titres of 1:160 or more. Lev (1982) is the only author who has found a complete disappearance of antibodies in two out of 6 experimentally infected rabbits. In his experiment, lasting for 68 days, 4 animals remained positive until the end of the experiment, while two lost their positivity on the 45th and 56th day, respectively.

The long-term persistence of antibodies (probably lifetime) demonstrated in our investigation is well in accordance with the chronic character of the disease. We suppose that in most cases the rabbits remain infected for life.

THE EPIDEMIOLOGY OF RABBIT ENCEPHALITOZOONOSIS

On two occasions we have investigated the transmission of EC as it occurs in conditions of intensive rabbit breeding at a farm.

In our first experiment (Vávra et al., 1978) we have introduced a group of 30 seronegative rabbits (N.Z. White and California White) into the hall of a farm with 50% seropositive animals of the same breed. These rabbits were caged in exactly the same manner as others and their cages were placed among those of the original rabbits. The rabbits were used for breeding and were fed and handled in the same manner as the rest of the animals in the hall. The sera of the rabbits were tested every 1-3 months for a period of 1 year. First serum conversion occurred after two months in one rabbit and since then the number of seropositive rabbits slowly increased. After one year, however, still about half of the rabbits maintained their original seronegativity.

In a second experiment (Vávra et al., unpublished) we placed one cage with a seropositive animal into the middle of a battery of wire cages containing 14 seronegative rabbits. All rabbits in this experiment were tested every 6 weeksfor a period of 9 months. Only two rabbits became seropositive during this period, one after 6 weeks, one after 4 and a half months. Both animals were housed in cages adjacent to the cage with the infected rabbit (one on the side, one below).

Both experiments show that the spread of encephalitozoonosis in conditios of a rabbit farm and in adult rabbits is very slow. This gives good prognosis for the prevention and the eradication of the parasite from commercial colonies.

The efficiency of EC transmission during rabbit breeding

This efficiency was tested over a two-year period in which 145 breeding does and their offsprings were serologically examined (Vávra et al. 1980). The young rabbits were examined when slaughtered (90 days). At this time they have lost the maternal EC antibodies which disappear at 4 weeks (Lyngset, 1980). In the first year of the experiment, young rabbits after weaning, were housed in large, common, outdoor cages irrespective of the serological status of their mothers. In the seceond year weaned offspring of seronegative and seropositive animals were caged separately. The results are shown in Tables 1 and 2 which demonstrate that significantly more seropositive animals are found among the offspring of seropositive does.

Table 1. Number of seropositive and seronegative rabbits born to seronegative does

	1st year (common caging)		2nd year (separate caging)	
	n	%	n	%
Positive	23	31	9	8
Negative	52	69	100	92

Table 2. Number of seropositive and seronegative rabbits born to seropositive does

	1st year (common caging)		2nd year (separate caging)	
	n	%	n	%
Positive	50	33	47	51
Negative	99	66	46	49

The difference was especially striking in the second year of investigation when only 8% of the young of seronegative does reacted positively (at 90 days of age) compared with 51% of rabbits born to seropositive does. This last number is strikingly similar to Lyngset's finding (1980) that 50% of

rabbits born to seropositive does seroconverted to positivity after the loss of maternal antibodies.

From our experiment, however, the way in which the young rabbit becomes infected remains obscure. Most of the infection might be the result of transmission by contamination. Transplacental transmission, however, cannot be ruled out. Although this mode of infection was described by Hunt et al. (1972), Owen and Gannon (1980) found that it was not a common mode of EC transmission.

The question arises why and how does the progeny of seronegative does become infected. The only way is due to infection by spores in the environment. This means that young rabbits must be particularly sensitive to EC infection in comparison to adult animals as the latter become infected relatively slowly (see above). Bywater and Kellett (1978) also suppose that rabbits up to the age of 5 months are particularly sensitive to EC infection.

THE INFLUENCE OF EC INFECTION ON RABBIT PERFORMANCE

EC is not very pathogenic and most cases in rabbits result in a mild, chronic disease without clinical signs.

Only Vávra et al. (1980) have examined the effects of chronic encephalitozoonosis on rabbit performance by comparing the average weight of meat carcasses of 426 rabbits (90 days old) in seropositive and seronegative animals. Our investigation was, however, complicated by frequent occurrence of heavy infections of the liver coccidian Eimeria stiedai (ES) which also influenced the weight of the rabbits. The results are shown in Table 3.

Table 3. The influence of the EC infection on the weight of meat carcass of 90 days old rabbits

	n	mean weight(g)	S.D.	%
A: Healthy (no EC and no ES)	130	1260	186.35	100
B: EC positive (but no ES)	40	1118.15	205.19	88.74
C: ES positive (but no EC)	167	1085.22	213.43	86.12
D: EC and ES positive	89	987.60	233.33	78.38

Our results clearly demonstrate that under normal conditions of farm breeding the weight of the meat carcass of EC positive animals is 11% lower than that of non-infected ones. ES infection alone decreases the weight of the meat carcass by 13%. The combination of both diseases has a profound effect on the meat performance of the rabbit causing the decrease of the weight of the meat carcass by 22%. These data show that both EC and ES are economically important parasites and that the attempts at the eradication of these parasites from rabbit farms is economically sound. It has also been reported that EC alters the immune responsiveness of the rabbit (Cox, 1977) but the importance of this phenomenon in practical rabbit breeding is unknown.

PREVENTION OF EC INFECTIONS IN RABBIT BREEDING

Evidently the most important measure is to prevent the entry of the parasite into the colony. When a rabbit colony is founded, the nucleus of breeding rabbits should be serologically tested twice during 2 months' quarantine. Also all animals introduced later to the colony should be first serologically tested under quarantine. All positive animals should be eliminated. The breeding rabbits should be occasionaly retested (especially the does). Only serologically negative rabbits should be sold to amateur breeders for starting their colonies.

In colonies in which EC is already established the most important measure is to test serologically all breeding animals and separate the negative ones (preferably into separate premises). Their respective progeny should also be kept separately. A systematic elimination of seropositive breeding animals and their replacement by negative ones should then follow. Occasional retesting of the breeding stock should be undertaken.

A high level of hygiene is also desirable, especially the efficient removal of urine and faeces. The availability of non-contaminated drinking water is also essential. Our experience shows that farms with good standard of hygiene show less infection with EC.

Such measures should be efficient as is shown by the successful elimination of EC from a SPF rabbit unit (Bywater and Kellett, 1978). In the case of progeny from genetically valuable but infected does, the method of Lyngset (1981) consisting of limiting the contact between the mother and its offspring, could be used.

The recommended measures might seem impracticable in the rabbit industry but we believe that a certain investment of time and money into an eradication programme might prove economically viable.

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COMPARISON OF SOME ISOLATION METHODS FOR RUMEN PROTOZOA

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Numerous species of ciliate protozoa can be found in rumen contents. An understanding of the significance of the protozoa in the rumen ecosystem can be gained from knowledge of their metabolic activities. The isolation of a single protozoal species from the rumen is usually necessary for the study of ciliate metabolism. The biochemical studies of the rumen protozoa are complicated by the presence of viable ingested and attached bacteria in the protozoal samples.

Several species of rumen entodiniomorphid protozoa have been grown successfully in vitro (Coleman 1971,1978), whereas the rumen holotrich protozoa are more difficult to cultivate for extended periods (Clarke and Hungate 1966). No rumen protozoon has been grown axenically (Coleman 1978). Cultures of entodiniomorphid protozoa must be fed every day and the maximum population density in the cultures is much lower than in the rumen (Coleman 1978). A selective cultivation medium is available only for Entodinium caudatum. It is therefore necessary, in order to isolate most entodiniomorphid protozoa, to pick out individual protozoa from rumen contents or a mixed culture in vitro (Coleman 1978). If Entodinium simplex is the only one of small Entodinium spp. (under 45 μm long) present in the rumen it can be separated by differential centrifugation.

Species that are difficult to culture in vitro have been isolated from conventional or partially defaunated rumen contents by either sedimentation, centrifugation or filtra-

tion techniques (Williams and Harfoot 1976; Williams and Yarlett 1982). The filtration with defined aperture Nylon textiles was used by Williams and Yarlett (1982) for the isolation of holotrich protozoa and by Gyulai (1983) and Williams et al. (1984) for the isolation of entodiniomorphid protozoa from monocultures and cocultures in the rumen. The purpose of this investigation was to compare some of the isolation methods used in combination with establishment of cultures *in vitro* or in the defaunated rumen.

MATERIALS AND METHODS

The ovine rumen was defaunated with Manoxol OT (BDH, Poole, England) by the procedure of Orpin (1977). *Ophryoscolex caudatus* was isolated from a conventional rumen with a micropipette attached to a micromanipulator. Individual ciliates have been sucked up from a drop of diluted rumen fluid which was placed on a slide under a microscope by the procedure of Coleman (1978). *E.simplex* was isolated by centrifugation at 600 g for 5 min and a drop of the supernatant fluid contained only this species (Coleman 1978).

The defaunated rumen was inoculated with *O.caudatus* and *E.simplex*. After 3 weeks a stable coculture developed in the rumen and the rumen fluid was used as a source of protozoa. However, it was necessary to keep the animal in isolation from faunated animals.

O.caudatus was separated from *E.simplex* by filtration through 56 µm aperture cloth (Simon Precision Textiles Ltd, Stockport, England). *O.caudatus* was retained by the filter and freed from bacterial and residual protozoa contamination by washing with an anaerobic, warm (39°C) buffer of the simplex-type (Coleman 1978). *E.simplex* was present in the filtrate and was separated from bacteria by centrifugation (600 g, 5 min). The surface area of filter was gassed with CO_2 and the usual precautions were observed to maintain anaerobiosis that is essential for the isolation of metabolically active protozoa.

E.simplex was cultivated *in vitro* by the methods of Cole-

man (1978). E.simplex was separated by filtration from O. caudatus in cocultures in vitro and monocultures of E.simplex were established.

RESULTS AND DISCUSSION

The limited protozoal population consisting of O.caudatus and E.simplex was stable for 7 months in the rumen of sheep and during this period no contaminating protozoal species was observed in the rumen. The population density of O.caudatus fluctuated between 5000-25000/ml and that of E.simplex between 700000-1000000/ml. Coleman and Reynolds (1982) have established O.caudatus in the defaunated rumen at a population density of 5000-12000/ml. The maximum population density of O.caudatus in culture was 125/ml (Coleman and Reynolds 1982).

The wide range and uniformity of the precision textiles commercially available offer considerable potential for the isolation of rumen protozoa on a size basis. The separation by filtration is efficient and easy from a suitable coculture in the rumen or in vitro but difficult from a complex protozoal population. However, Williams and Yarlett (1982) separated Dasytricha ruminantium from a conventional rumen by the sequential use of 45,30 and 15 μm aperture cloths.

Differential centrifugation separates completely only the smallest ciliates present in the rumen. The required species of protozoa can be picked up with a micropipette attached to a micromanipulator from rumen contents and inoculated into a growth medium or a defaunated rumen. Coleman (1978) described the 2 main difficulties involved as /a/ the necessity for speed because the protozoa are strict anaerobes and are exposed to air during the procedure and /b/ the risk of removing small Entodinium spp. along with the larger protozoon that is isolated.

This investigation has shown the advantage of the separation by filtration if a suitable coculture of rumen protozoa with considerable size differences is available.

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ADVANCES IN VETERINARIAN PARASITIC PROTOZOA

II. CHEMOTHERAPY OF PROTOZOAN DISEASES

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CHEMICAL CLASSIFICATION OF ANTICOCCIDIALS AND THEIR REPLACEMENT: A REPORT

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Every drug is followed by its "shadow-resistance" which increases simultaneously with each subsequent passage of the parasite. In the case of coccidia this resistance increases especially rapidly. I take the courage to conclude that coccidia occupy the first place among all the pathogens in this respect.

Today, we can only state the existence of this resistance. Some chemicals, e.g. acriflavine, reverse the susceptibility of coccidia to the inhibitor a little, but are not applicable in practice because of their mutagenicity. The only way of solving this problem is replacing the drug by another one. I shall attempt to outline the principle. I suggest that if two drugs have the same biochemical mode of action on a parasite, the biochemical mode of parasite resistance against them will also be identical, and vice versa. In my opinion, resistance is not else but a struggle of the parasite against the mechanisms of drug action on it. If the mechanisms of action are identical, the mechanisms of counteraction, i.e. resistance, must be identical too. That is why many authors note that the resistance of coccidia to drugs of one type has a group character.

The biochemical mechanism of action, as every property of a chemical molecule, depends on its chemical structure. In this respect I propose the following classification of anticoccidials (see Table 1).

Table 1

CLASSIFICATION OF ANTICOCCIDIALS

DRUGS FOR BROILERS AND LAYING HENS

Thiamine pyrophosphokinase inhibitor 1

1. Amprolium

Dihydrofolate-synthetase inhibitors 2-6

2. Sulphachlorpyrazine
3. Sulphadimidine
4. Sulphadimetoxine
5. Sulphamonometoxine
6. Sulphaquinoxaline

Reduced flavine- and cystine-containing enzyme inhibitors; derivatives of 5--nitrofurane and 3,5-dinitrobenzamide 7-9

7. Zoalene
8. Iramine
9. Furazolidone

Ornithinedecarboxylase inhibitor 10

10. d,l-Difluormethylornithine

Anticoccidial mixtures 23-31

23. Amaidon Punch: 4+9+16
24. Darvisul: 6+Diaveridine
25. Saguadil: 6+Diaveridine
26. Rofeneid: 4+Ormethoprim
27. Rofenon: 4+Diaveridine
28. Duococcin: 1+6
29. Pancoxine: 1+6++Etophalate
30. Lerbek: 14+17
31. Rigecostat: 12++17

DRUGS FOR BROILERS

Purine analogs 11-16

11. Arprinocid
12. Buquinolate
13. Deccoquinate
14. Methylbenzoquate
15. Halofuginone
16. Glycarbylamide

Pyrimidine analog 17

17. Methylchlorpindol

Monoaminooxidase and acetylchalinesterase inhibitor, calciumtrop 18-19

18. Robenidine
19. Himcoccid

Inhibitors of normal transport of ions through the biological membranes (ionophores) 20-22

20. Lasalocid
21. Monensin
22. Salinomycin

If we replace the first drug, which belongs to one chemical class, by another one, it is necessary to choose the second drug from another class. Table 2 shows the scheme of substitution.

Table 2

REPLACEMENT OF ANTICOCCIDIALS

The drugs which must be replaced because of parasite resistance	What drugs can be replaced for
1*	2-27, 30, 31
2-6, 24-27	1, 7-23, 28-31
7-9	1-6, 10-31
10	1-9, 11-31
11-16	1-10, 17-31
17	1-16, 18-31
18, 19	1-17, 20-31
20-22	1-19, 23-31
23	1, 17-22, 24-31
28	7-23, 30, 31
29	7-23, 30, 31
30, 31	1-10, 18-29

*1 - Amprolium, 2 - Sulphachlorpyrazine, 3 - Sulphadimidine, 4 - Sulphadimetoxine, 5 - Sulphamonometoxine, 6 - Sulphaquinoxaline, 7 - Zoalene, 8 - Iramine, 9 - Furazolidone, 10 - d,l-Difluormethylornithine, 11 - Arprinocid, 12 - Buquinolate, 13 - Deccoquinate, 14 - Methylbenzoquate, 15 - Halofuginone, 16 - Glycarbylamide, 17 - Methylchlorpindol, 18 - Robenidine, 19 - Himcoccid, 20 - Lasalocid, 21 - Monensin, 22 - Salinomycin, 23 - Amaidon Punch, 24 - Darvisul, 25 - Saguadil, 26 - Rofeneid, 27 - Rofenon, 28 - Duococcin, 29 - Pancoxine, 30 - Lerbek, 31 - Rigecostat

This principle has already been published by us (Kozhemiaka et al., 1984). Besides, my colleagues and I have made an attempt to give a mathematical model which allows calculation

of the amount of time during which an anticoccidial can be used in poultry farming. The model is based on laboratory data, given in passages (Gutschabash et al., 1984).
While introducing new and new drugs in poultry farming, we contaminate nature. When resistance will be overcome, we shall stop this contamination with pleasure.

SUMMARY

The only way of solving the problem of resistance of coccidia to anticoccidials is replacement of one drug by another, the latter being chosen from another chemical class. The anticoccidials are divided into the following classes: Enzyme inhibitors: (1) thiamine pyrophosphokinase inhibitor, (2) dihydrofolate-synthetase inhibitor, (3) reduced flavine and cystine-containing enzyme inhibitors, (4) monoaminooxidase and acetylcholinesterase inhibitor, calciumtrop, (5) inhibitors of normal transport of ions; Analogs: (6) purine analogs, (7) pyrimidine analog, (8) anticoccidial mixtures.

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ANTICOCCIDIAL IONOPHORES: NEW PROSPECTS IN THE CONTROL OF BROILER CHICK COCCIDIOSIS

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Anticoccidial ionophores possess a variety of properties not shared by coccidiostatic agents; thus, they open up new vistas in the control of broiler chick coccidiosis. First of all, these compounds have a coccidiocidal effect which, under certain management technological conditions, would make possible the prolonged use of the same deep litter and, thereby, save fuel in our continental climatic conditions. The different Coccidium species develop no resistance to ionophores, or resistance develops only after very prolonged use (Chapman, 1976; Jeffers, 1981; Mitrovič and Schildknecht, 1975). Finally, in some feeding conditions, ionophores enhance the body mass gain and improve the feed conversion of the chicks. It is no mere chance that, since its discovery in 1967, monensin has made such an enormous career: after more than 15 years of use, in the United States it still dominates more than 80 % of the market of anticoccidial drugs used against chick coccidiosis.

Since that time the number of anticoccidial ionophores has increased, because the following ionophores have been developed, in chronological order: salinomycin (1973), lasalocid (1975) and narasin (1976). Other ionophores currently under experimentation are carriomycin, lonomycin and septamycin. Of these drugs monensin, lasalocid and salinomycin are in circulation in Hungary.

A disadvantageous property of the ionophores is that they are rather toxic and, by exerting their effect on the very young

developmental stages of the parasite, they hinder the development of immunity (Karlsson and Reid, 1978).

The ionophores prepared from different fungus species by fermentation are very similar as regards their mode of action, viz. they facilitate the transport of K^+, Na^+, and occasionally also Mg^{++} and Ca^{++} ions through the cell membranes, but differ from one another in a whole series of properties. With the exception of lasalocid, ionophores promote the transport of only the monovalent alkaline metal ions, whereas lasalocid furthers the transport of bivalent cations (Mg^{++} and Ca^{++}) as well. Obviously this is why in laboratory trials, in very massive experimental invasion (100 to 200 thousand sporulated oocysts), the recommended dose of lasalocid prevents the consequences of coccidiosis, whereas the ionophores promoting the transport of only the two monovalent ions (monensin, salinomycin) fail to do so (Bains, 1980; Varga, 1979, 1980, 1984; Visnyei, 1983). Lasalocid-treated chicks show the best body mass gain despite the fact that occasionally such chicks develop more lesions attributable to coccidia than chicks fed monensin or salinomycin (Bains, 1980; Bedrník et al., 1979; Chappell and Babcock, 1979; Frigg and Streif, 1980; Hera et al., 1979; McDougald and McQuistion, 1980; McDougald, 1981; Migaki et al., 1979; Riedl and Kützer, 1981; Udvarhelyi and Visnyei, 1980). This was proved also by studies (Weppelmann et al., 1977) in which the sensitivity of different strains of Coccidium species to monensin, lasalocid and salinomycin was tested. Namely, lasalocid was effective against a variety of strains which were refractory to monensin and salinomycin. However, only one strain was found on which lasalocid exerted a weak effect and which was sensitive to monensin and salinomycin.

Since the coccidial ionophores are toxic, the recommended doses (monensin 100 ppm, lasalocid 75 ppm, salinomycin 60 ppm) must be strictly observed, otherwise the body mass gain of broilers will be reduced. According to data of the literature, monensin seems to be the most, while lasalocid the least toxic (Damron et al., 1977; Frigg and Streif, 1980; McDougald and McQuistion, 1980). A threefold overdosage of

monensin may lead to death, a twofold overdosage of salinomycin may reduce the body mass gain by approximately 40 %, while a more than 2 1/2-fold overdosage of lasalocid results in barely any reduction of the body mass gain. Turkeys are particularly sensitive to monensin and salinomycin (Davis, 1983; Frigg et al., 1983; Stuart, 1978, 1983).

In chicks fed monensin, after the withdrawal of the drug a so-called compensatory growth can be observed for a week: such a phenomenon was not seen among chicks fed other anticoccidial drugs (McDougald, 1980).

Several authors (Friesecke et al., 1977; Patel et al., 1979, 1980; Ward and Brewer, 1980; Yamane et al., 1980) have studied the effect of sulphur-bearing amino acids, particularly methionine, in the presence of anticoccidial ionophores. These studies have shown that the use of lasalocid causes no feathering disturbances even if only about 33 % of the prescribed methionine dose is present in the feed.

Repeated investigations (Damron et al., 1977; Frigg et al., 1980; Marusich et al., 1977; Mitrovic et al., 1975) have dealt with the incompatibility of different growth-promoting substances (roxarsone, zinc-bacitracin, lincomycin) or different antibiotics with the various anticoccidial ionophores. These investigations have revealed that monensin is incompatible with tiamulin, erythromycin, a variety of sulphanylamides and furidine, salinomycin is incompatible with tiamulin and kitamasin, while lasalocid is compatible with all the substances tested.

Results of recent investigations made in Hungary (Stipkovits et al., 1984) allow us to conclude that lasalocid exerts a strong bacteriostatic and bactericidal effect on Mycoplasma gallisepticum as well. After the results of preliminary "in vitro" experiments, this fact was confirmed also by an "in vivo" experiment. In the latter, 50 three weeks old roosters assigned to five groups of equal size were used. The five groups were as follows: (1) infected, not treated; (2) non-infected, treated with lasalocid; (3) infected, treated with lasalocid; (4) infected, treated with tylan; (5) non-infected, not treated.

The birds were inoculated intraperitoneally with 0.3 ml of a mixture of the broth culture of three M. gallisepticum strains (designated 8318, 8400, M516). Lasalocid and tylan were added to the feed in concentrations of 75 and 100 ppm, respectively, and the medicated feed was fed for 5 days as from the day of inoculation. The inoculated chicks were kept under observation for 3 weeks and their body mass gain was recorded individually at the end of each week. The results were evaluated on the basis of lesions developed in the respiratory tract and in the peritoneum, and the body mass gain. Of the 10 non-treated, infected chicks 70 % showed lesions; the proportion of such birds in the infected but tylan- or lasalocid-treated groups was 20 and 30 %, respectively. The body mass gain of chicks in the different groups was as follows: infected, tylan-treated: 978 g; non-infected, lasalocid-treated: 891 g; infected, lasalocid-treated: 878 g; non-infected, not treated: 823 g; infected, not treated: 733 g.

Table 1

EFFICACY OF LASALOCID-Na AGAINST M. GALLISEPTICUM IN EXPERIMENTALLY INOCULATED CHICKS

Group	Treatment	Inoculation	Weight gain (g) in the				Pathological alterations*
			1st week	2nd week	3rd week	Total of experiment	
1	-	$\bar{x}$	247	310	176	733	
	Not treated	+ s	18	29	27	7	7/10
2	Lasalocid-Na	$\bar{x}$	303	325	263	891	
	75 mg/kg	+ s	50	13	6	32	0/10
3	Lasalocid-Na	$\bar{x}$	250	332	296	878	
	75 mg/kg	+ s	54	15	8	8	3/10
4	Tylan	$\bar{x}$	323	341	314	978	
	100 mg/kg	+ s	16	17	38	14	2/10
5	-	$\bar{x}$	263	309	251	823	
	not treated	+ s	10	8	10	10	0/10

*No. of chicks with alterations/No. of chicks in the group.

SUMMARY

Of the anticoccidial ionophores, the author discusses those properties of monensin, lasalocid and salinomycin through which these drugs open up new vistas in industrial-scale broiler production.

The properties by which the above three compounds can be distinguished from one another are also reviewed.

Namely, lasalocid is more efficacious in preventing the consequences of a very massive single invasion than monensin or salinomycin, which fact is obviously due to its property that it is able to transport through cell membranes not only the monovalent Na^+ and K^+ ions but the bivalent Ca^{++} and Mg^{++} ions as well.

Lasalocid is less toxic than monensin and salinomycin, and it prevents the development of the consequences of a partial deficiency in sulphur-bearing essential amino acids, especially methionine.

Lasalocid is compatible with a number of antibiotics, among others with tiamulin, with which monensin and salinomycin are incompatible.

Finally, the results of Hungarian experiments are reported, according to which lasalocid protects the chicks against the pathogenic effects of different Mycoplasma gallisepticum strains.

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STUDIES ON THE PROBLEMS OF RESISTANCE AND CROSS-RESISTANCE WITH IONOPHORE ANTICOCCIDIALS IN CHICKS

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INTRODUCTION

One of the essential problems in antiparasitic prophylaxis and therapy of farm animals is the ability of parasites to rapidly develop resistance against new drugs. With regard to coccidiosis control in chicks, a complete resistance against almost all synthetic anticoccidial products was observed in the field (Jeffers, 1974; Mathis and McDougald, 1982; Braunius et al., 1984) and was successfully developed under laboratory conditions (McLoughlin and Chute, 1975; Chapman, 1978). In contrast, the ionophore anticoccidials are still active against coccidiosis, even when used intensively for many years in the field. Several attempts to induce resistance against ionophore anticoccidials failed (Mitrovič and Schildknecht, 1975; Chapman, 1976; Jeffers, 1981). In a recent laboratory experiment coccidia, which had been propagated 5 times in medicated chicks, were even more susceptible to lasalocid than the parent strain (Weber and Frigg, 1985). However, Chapman (1984) successfully developed resistance in Eimeria tenella against monensin after 16 passages in medicated chicks.

Because of the outstanding efficacy of ionophores against coccidiosis, many products of this group (monensin, lasalocid, salinomycin, narasin, prinicin) with similar chemical structure and mode of action are currently on the market. Therefore, the question of a possible cross-resistance among these products is of eminent interest. Obviously, between ionophores

and synthetic anticoccidials no cross-resistance occurs (Mitrovič and Schildknecht, 1975; Jeffers, 1981). However, some indications for cross-resistance between monensin, narasin and salinomycin have been reported by Weppelman et al. (1977) and Jeffers (1984).

The aim of the present study was to investigate a possible development of resistance of coccidia against lasalocid and monensin when 2 strains, originally derived from problem field isolates, were passaged repeatedly in medicated chicks. Further, the occurrence of cross-resistance among 3 ionophore anticoccidials was examined by testing lasalocid and salinomycin against that sample of coccidia which had been hardly controlled by monensin.

MATERIALS AND METHODS

In experiment 1 broiler chicks ("Mini-Hubbard") reared on a commercial maize-wheat-soybean meal diet (crude protein 21.7 %, metabolizable energy 13.2 MJ) were used. From day 10, the chicks were kept in wire-floored cages and fed the basal diet supplemented with either 125 ppm lasalocid or 120 ppm monensin. The control chicks (UUC = uninfected unmedicated control; IUC = infected unmedicated control) received the basal diet without an anticoccidial drug. Each treatment was replicated 3 times (UUC: 6 times) with groups of 4 chicks each (2 females, 2 males). On day 16 the respective chicks were inoculated orally with 250,000 oocysts of 4 coccidium samples., originally derived from 2 field isolates (725: from a farm using lasalocid; 744: from a farm using monensin). Both isolates contained a mixture of Eimeria acervulina and E. tenella. These coccidia had been maintained for some time in the laboratory and had been propagated 3 times prior to this experiment in chicks medicated either with 150 ppm lasalocid (725/LAS3; 744/LAS3) or 150 ppm monensin (725/MON3; 744/MON3). Weight gain and feed conversion were regularly measured during the experiment. On day 23, the chicks were sacrificed and scored for gross lesions (1-3 = slight to severe lesions, 4 = died from coccidiosis). Oocyst production during the last

2 days of the infective cycle was determined by counting the oocysts excreted in the faeces.
Experiment 2 was conducted with broiler chicks ("Vedette") fed the same diet as in experiment 1. Groups of 6 chicks (3 females, 3 males each) were formed and kept in cages on the basal diet, supplemented either with 125 ppm lasalocid, with 120 ppm monensin, or with 60 ppm salinomycin from day 10 onwards. Each experimental treatment as well as the negative control (IUC) was replicated 5 times. The positive control (UUC) consisted of 7 groups. On day 16, the chicks were inoculated orally with 250,000 oocysts of strain 744, which had previously been propagated 2 times in chicks medicated with 150 ppm monensin. On day 23, the chicks were sacrificed and the same parameters as in experiment 1 were recorded.

RESULTS

Experiment 1 (see Table 1)

The coccidia of strain 725, propagated under lasalocid medication (725/LAS3), were less pathogenic than those from the monensin propagation (725/MON3) as shown by performance and mortality of the IUC. With coccidia 725/LAS3 more lesions were observed in the small intestine (E. acervulina) and more oocysts were found in the faeces than with sample 725/MON3. The latter coccidia (725/MON3), however, caused more severe caecal lesions (E. tenella). Lasalocid was active against both samples of strain 725 regarding performance and lesion score. Monensin controlled sufficiently the sample 725/LAS3, but exhibited only a moderate activity against those coccidia which had been propagated in monensin-medicated chicks (725/MON3).
In general, coccidia of strain 744 were more pathogenic than those of strain 725. With the coccidia 744/LAS3 a higher mortality was noted than with 744/MON3 in both the IUC and the monensin-medicated chicks. Monensin failed to control the sample designated 744/LAS3 and exhibited only a weak activity against coccidia 744/MON3. Medication with lasalocid, however, allowed a normal performance and protected the chicks from mortality and severe caecal lesions.

Table 1

PERFORMANCE AND PARASITOLOGICAL PARAMETERS OF CHICKS, INOCULATED ON DAY 16 WITH COCCIDIA OF TWO STRAINS (725, 744) WHICH HAD BEEN PROPAGATED 3 TIMES IN LASALOCID- (725/LAS3, 744/LAS3) OR MONENSIN- (725/MON3, 744/MON3) MEDICATED CHICKS (EXPERIMENT 1)

Treatment		Mean weight gain (g/chick/day) Day 16-23 (2)	Mean feed conversion Day 16-23 (2)	Lesions in		Oocyst production per chick per day x 10^6	Mortality (in %) Day 16-23
Sample	Anticoccidial (1)			small intestine	caeca		
725/LAS3	UUC	42.4 A	1.66 C	0	0	<0.2	0
	Lasalocid 125 ppm	36.1 B	1.91 B	0.1	0.6	142.7	0
	Monensin 120 ppm	31.8 C	2.02 B	0	1.0	116.6	0
	IUC	25.3 D	2.36 A	1.9	2.4	240.3	0
725/MON3	UUC	42.4 A	1.66(3)	0	0	<0.2	0
	Lasalocid 125 ppm	36.5 A	1.82	0.1	1.5	127.7	0
	Monensin 120 ppm	23.2 B	2.50	0.1	2.5	91.0	0
	IUC	7.8 C	24.66	0.6	2.9	124.9	33
744/LAS3	UUC	42.4 A	1.66 B	0	0	<0.2	0
	Lasalocid 125 ppm	35.6 B	1.93 B	0	1.3	120.0	0
	Monensin 120 ppm	18.6 C	2.92 AB	0	2.7	156.1	17
	IUC	11.1 D	4.75 A	0.3	3.8	72.0	67
744/MON3	UUC	42.4 A	1.66 C	0	0	<0.2	0
	Lasalocid 125 ppm	40.6 A	1.68 C	0	1.8	33.7	0
	Monensin 120 ppm	28.8 B	2.08 B	0.1	2.6	64.1	8
	IUC	15.3 C	3.33 A	0.2	3.0	151.0	17

(1) UUC: mean of 6 groups, infected treatments: mean of 3 groups with 4 chicks each

(2) Duncan test: means within one field, not followed by a common letter, are significantly different ($\alpha < 0.05$)

(3) Duncan test not done

Experiment 2 (see Table 2)

As in experiment 1, monensin failed to control coccidia of strain 744 which had been propagated 2 times under this drug. Based on weight gain (WG), the relative efficacy (RE = = ((WG'MON' - WG'IUC')/(WG'UUC' - WG'IUC')) x 100) of monensin was only 28 %. Moreover, severe lesions were found in the caeca and a considerable mortality occurred. The performance of chicks medicated with salinomycin was significantly superior to that with monensin, but compared with the UUC the efficacy (RE = 46 %) was low. However, salinomycin remarkably reduced caecal lesions and oocyst production. Lasalocid showed a considerable activity not only in terms of performance (RE = 71 %), but also with regard to lesion score and mortality.

Table 2

PERFORMANCE AND PARASITOLOGICAL PARAMETERS OF CHICKS, INOCULATED ON DAY 16 WITH COCCIDIA OF STRAIN 744, WHICH HAD BEEN PROPAGATED 2 TIMES IN MONENSIN-MEDICATED CHICKS (EXPERIMENT 2)

Treatment	Number of groups x 6 chicks	Mean weight gain (g/chick/day) Day 16-23 (*)	Mean feed conversion Day 16-23 (*)	Lesions in Sm.i.	Lesions in c.	Oocyst production per chick per day x 10^6	Mortality (in %)
UUC	7	40.0 A	1.60 C	0	0	<0.1	0
Lasalocid 125 ppm	5	32.4 B	1.77 C	0	1.4	48.0	0
Monensin 120 ppm	5	20.8 D	2.50 B	0	2.5	57.7	10
Salinomycin 60 ppm	5	25.7 C	1.97 C	0	1.1	17.6	0
IUC	5	13.4 E	3.47 A	0	3.3	69.5	37

(*) Duncan test: means, not followed by a common letter, are significantly different ($\alpha < 0.05$). Sm. i.=small intestine, c. = caeca

DISCUSSION

In experiment 1 a possible development of resistance of coccidia was investigated under laboratory conditions. Two ionophore anticoccidials, to which the parasites had been intensively exposed in the field, were studied. It was found that lasalocid controlled coccidia of both strains and that previous repeated passages of the parasites under lasalocid medication did not impair their sensitivity to this drug. These results confirmed earlier observations of Mitrovič and Schildknecht (1975). In contrast, coccidia of sample 725, propagated under monensin (725/MON3), were less sensitive to monensin than those of 725/LAS3. These findings indicated a development of a partial resistance of coccidia 725/MON3 against monensin during the propagations, accompanied by an increased pathogenicity of that sample. Monensin nearly failed to control coccidia of strain 744 exposed previously to monensin in the field. However, compared to the results with sample/LAS3, no further reduction in sensitivity of coccidia to monensin occurred when the parasites were propagated under that drug (744/MON3). In contrast to the findings of Chapman (1984), a restoration of sensitivity of coccidia 744/LAS3 to monensin was not observed, even though they were exposed during 3 passages to lasalocid. Since strain 744 was very pathogenic too, in agreement with Chapman (1976) the development of resistance seemed to be linked with an increase in the pathogenicity of coccidia.

In experiment 2 strain 744 was propagated 2 times under monensin prior to the experiment and was tested against monensin, lasalocid, and salinomycin. Monensin again hardly controlled these coccidia, with regard to both performance and parasitological parameters. These poor results, comparable to the observations of Chapman (1984), confirmed this to be a severe case of partial resistance against monensin. This resistance could be based on an erosion of anticoccidial potency, due to intensive use of monensin in the field and during the passages in the laboratory. Whereas lasalocid was found to be active enough to efficiently control these highly pathogenic coccidia, the efficacy of salinomycin regarding

chick performance was judged to be only moderate. Taking into account that in this experiment these coccidia were exposed for the first time to lasalocid and salinomycin, it is probable that the poor efficacy of salinomycin was due to a certain cross-resistance between this drug and monensin.
Our results agree with earlier findings of Jeffers (1984) who reported highly significant correlated responses of monensin, salinomycin and narasin to different strains of E. tenella and discussed his results as being an indication for a cross-resistance among those drugs. Since monensin and salinomycin are members of the same group of the monovalent ionophores (Westley, 1982), it could be assumed that the observed cross-resistance was based on the closely related chemical structure of these two drugs. Lasalocid, however, is a divalent ionophore (Westley, 1982) differing from the monovalent drugs in structure and mode of action. Thus, the risk is rather low that coccidia resistant to one of the monovalent anticoccidials would simultaneously show resistance to lasalocid.

SUMMARY

A strain of mixed Eimeria species, which had been intensively exposed to the ionophore anticoccidials lasalocid and monensin, was found to be partially resistant to monensin. With another strain of coccidia, the sample propagated under monensin medication was less sensitive to monensin than the sample passaged with lasalocid. In general, lasalocid controlled all of these coccidium samples. A certain cross-resistance was observed between monensin and salinomycin.

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COMPARATIVE STUDY OF THE EFFICACY OF IONOPHORE ANTICOCCIDIAL ANTIBIOTICS

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The problem of poultry coccidiosis control has maintained its timeliness up to the present day. This can be attributed to the fact that the various Eimeria species develop resistance against the applied anticoccidials within a very short time, resulting in a rapid decrease of efficacy of these anticoccidials and, consequently, severe economic losses. Economic losses are made up of deaths, reduced productivity (meat and egg production), and impaired feed utilization.

Recently, ionophore antibiotics have been used more and more widely to control coccidioses.

In the present work, we compared the anticoccidial efficacy of Avatec (lasalocid), Sacox (salinomycin), and Elancoban (monensin) under laboratory and field conditions.

Under laboratory conditions, Avatec and Sacox applied at the recommended dose rate consistently prevented the development of coccidiosis after a mixed (E. tenella, E. acervulina, E. maxima) experimental infection of an LD_{50}-LD_{70} dose rate. Among chicks medicated with Elancoban, 10 to 20 % mortality occurred.

Chicks medicated with Avatec showed considerably higher body mass gain than those treated with Sacox or Elancoban (by 27.6 and 49.4 %, respectively). The anticoccidial indices of the latter two drugs were by 21.5 and 38.5 points worse, respectively, than those of Avatec.

Toxicity studies conducted with the preparations have revealed that salinomycin and, especially, monensin are toxic com-

pounds, and even their slight overdosage results in considerably poorer body mass gain of the chicks.

The results of field trials indicate that, when given in the feed in the recommended concentrations (0.5 and 0.1 %, respectively), under our conditions of production both Avatec and Sacox surely prevent the development of clinical coccidiosis throughout the entire broiler-rearing period, and ensure normal growth and body mass gain of the birds.

According to the results of trials conducted with Elancoban, under our conditions of production a 0.1 % concentration of Elancoban in the feed does not fully prevent the development of coccidiosis: from 47 days of age onwards, clinical coccidiosis occurred regularly, accompanied by deaths and the gross pathological picture characteristic of coccidiosis. To prevent further deaths, these stocks had to be treated with Coccidiovit given via the drinking water in a dose of 1 g/l.

Under the conditions of production, feed costs were practically identical in the case of Avatec- and Sacox-medication; however, as compared to Elancoban-treated birds, Avatec- and Sacox-medicated chicks required by 0.58 and 0.79 feed units less, respectively, for one unit of body mass gain.

Based upon the results of our laboratory experiments and field trials it can be established that monensin is not too promising as a prophylactic compound of broiler coccidiosis.

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EFFECTIVENESS OF SEVERAL ANTICOCCIDIAL DRUGS IN PREVENTING COCCIDIOSIS IN CHICKENS REARED FOR LAYERS

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Prevention of coccidiosis in chickens reared for layers, kept on litter, is mainly based on chemoprophylaxis. The attempts made to immunize the birds against the disease actively are still of organic character and not fully effective (Edgar, 1958; Jeffers, 1975; Johnson and Reid, 1979; Long, 1984; Long and Millard, 1977; Long and Rose, 1982; McDonald and Ballingall, 1983; McDonald et al., 1982; Rose, 1976; Sanda, 1985; Shirley et al., 1984).

The development of immunity against coccidiosis in birds reared for layers depends on the type and dose of anticoccidials applied, the health status of birds, the standard of feeding and management. In chickens raised for layers, mainly such coccidiostats are recommended which do not set back the development of schizonts of the second generation responsible for developing immunity against coccidiosis (Reid et al., 1976). According to Colnago et al. (1984), the proper level of vitamin E and selenium in the feed has a positive influence on the development of anticoccidial immunity, whereas viral infections of birds, in particular Marek's disease virus (Blount, 1954; Getler, 1974), infectious bursal disease virus as well as increased levels of ammonia in chicken houses (Quarles and Fagerberg, 1979) have an unfavourable bearing on immunity.

The present paper reports the results of studies carried out on chickens reared for layers in which different anticoccidials were applied in a shuttle-programme, in order to develop immunity against coccidiosis.

MATERIALS AND METHODS

The studies were performed in 9 standard chicken houses (standard design for brick houses) with an area of 300 to 960 m^2 available for the birds. The starting number of birds was 3600 to 8200 chicks with a density of 6.3 to 19.5 birds per m^2 at the initial stage of rearing. In the 20th week of the birds' life there were 4.5 to 7.7 birds per m^2. In chicken houses No. I, VII, VIII and IX there was straw litter, whereas in chicken houses No. II, III, IV, V and VI sawdust was used. All of the houses were equipped with standard feeding and drinking devices. Ventilation was installed in the walls and ceiling. Windows and artificial lighting (incandescent) provided the essential amount of light.

The birds were fed standard nutritive mixtures, i.e. DKM-1 (from the 1st up to the 8th week), DKM-2 (9th-20th week), and DJ-1 (from the 21st week up to the end of the production cycle). Birds accommodated in chicken houses No. I-V the following anticoccidials were administered in a shuttle programme:

- Avatec - 75 ppm/ Amprol Plus - 133 ppm;
- Coyden 25 - 125 ppm/ Amprol Plus - 133 ppm;
- Cycostat - 33 ppm/ Amprol Plus - 133 ppm;
- Elancoban - 90 ppm/ Amprol Plus - 133 ppm;
- Lerbek - 110 ppm/ Amprol Plus - 133 ppm.

The first anticoccidial mentioned was administered during the first 8 weeks, while the second one from the 9th week up to the 20th week of rearing. In the remaining 4 groups of the birds (No. VI-IX), i.e. the controls, Amprol Plus (133 ppm) was applied throughout the period of raising according to the obligatory domestic prophylactic programme.

In the course of rearing, the zoohygienic status of chicken houses, the birds' health and the effects exerted on production were evaluated. The zoohygienic studies included measurement of thermic and humidity factors, cooling, air circulation, concentration of NH_3 and CO_2, dustiness of the air, in-

tensity of lighting and ventilation as well as germ count of the air. These measurements were made according to the methods generally recognized, at one-month intervals (5 series of tests), before noon, in 3 constant places of the chicken houses. The basic physical and chemical parameters of litter, i.e. temperature, water content, relative humidity, concentration of NH_3 and CO_2, were measured too. These measurements and microclimatic studies were performed simultaneously, at a depth of 5 cm from the surface of litter.

In the 4th, 8th, 12th and 20th week of rearing, 6 birds chosen at random from each chicken house were examined parasitologically for the presence of coccidiosis. At the time mentioned above and, additionally, in the 24th week, litter samples were taken from each chicken house to determine their contamination with oocysts (Long and Millard, 1977). Moreover, in the 24th week, 8 birds were chosen at random from each chicken house for challenge infection to estimate immunity of the birds against coccidiosis. After a 7-day adaptation in cages, the birds were infected experimentally with a mixed culture of sporulated oocysts of E. acervulina (250,000), E. necatrix (50,000) and E. tenella (150,000). On the 8th day after infection with Eimeria spp. the birds were killed to examine pathological changes in the alimentary tract. Evaluation of the birds' immunity against coccidiosis comprised the following parameters: body weight, feed consumption, survival rate, oocyst production (to determine the number of oocysts, the faeces was collected during the last 24 hours of the test) and pathological changes in the alimentary tract (determined according to Johnson and Reid, 1970).

The health status of birds in each group was examined by routine diagnostic investigation of the dead birds, serological examination of 20 blood samples chosen at random from each chicken house to demonstrate viral infections (infectious bursal disease (IBD), infectious bronchitis (IB), infectious laryngotracheitis (ILT), reovirus and adenovirus) and Mycoplasma gallisepticum. Thigh feathers were collected from the same birds to investigate the presence of Marek's disease virus (MDV). Blood sera were examined for viral in-

fections by the immunodiffusion method in agar gel and for M. gallisepticum by the tube agglutination test. To examine birds for infection with MDV, the radial immunodiffusion method using feather tips and immune serum from chicks infected with MDV was applied.

RESULTS

The results of zoohygienic studies have shown that the climatic conditions of houses and the qualitative parameters of the litter used in the chicken houses examined varied insignificantly. The concentration of CO_2 and NH_3 did not exceed 0.2 % and 13 ppm, respectively, i.e. the standard limit for these birds. The litter was characterized by significant thermic-humidity autonomy. Some variations in the type of litter used in the chicken houses were found.

Generally, the results obtained (Table 1) in the 9 chicken houses studied are within, or approximate, the domestic technological standards. The overstandard losses found on poultry farms No. II ($\bar{x}$ = 18.01 %), No. V ($\bar{x}$ = 11.31 %) and No. VI ($\bar{x}$ = 12.24 %) should be underlined. On the above-mentioned farms most of the losses took place during the 1st month of rearing. On farm II they resulted from aspergillosis, salmonellosis, colibacillosis, whereas on farm V from omphalitis, yolk sac inflammation and colibacillosis. On farm VI, diathesis urica and colibacillosis were responsible for the losses. In the production period, overstandard losses took place only on farm No. VI ($\bar{x}$ = 1.02 %) and VIII ($\bar{x}$ = 1.69 % monthly). On farm VI they were due to colibacillosis and on farm VIII they came from fatty liver dystrophy and mycotoxicosis.

Serological examination of blood samples and feather tips (Table 2) did not reveal an infection of birds with IB virus, ILT virus and MDV. In 5 flocks IBD infection was demonstrated, adenovirus and reovirus infection appeared in 2 flocks, while in 7 flocks M. gallisepticum infection was observed. The last one caused no clinical symptoms.

Table 1

PRODUCTION PARAMETERS OF THE POULTRY FARMS

Poultry farm	Anticoccidial preparations administered and their dose*	Strain of birds	Number of birds	Body weight in the 20th week (g)
I-"KR"	Avatec-75 ppm/ /Amprol Plus - - 133 ppm	Astra-229	8181	1800
II- -"SP"	Coyden-125 ppm/ /Amprol Plus- 133 ppm	C-89	6074	2090
III- -"DJ"	Cycostat-33 ppm Amprol Plus-133 ppm	Astra-S	3570	2030
IV- -"PW"	Elancoban-90 ppm/Amprol Plus -133 ppm	C-89	6095	2040
V-"GJ"	Lerbek-108 ppm/ Amprol Plus-133 ppm	C-89	5924	2030
VI- -"SJ"	Amprol Plus- 133 ppm - control	W-74	3947	2300
VII- -"MJ"	Amprol Plus- 133 ppm - control	W-74	6362	2010
VIII- -RSP"J"	Amprol Plus- 133 ppm - control	Lg	6000	1037
IX-"DJ"	Amprol Plus - 133 ppm	Astra S	3620	2050

Explanation: *expressed as active compound

Table 1 continued

Feed consumption during 20 weeks (kg)	Losses and condemnation during 20 weeks (%)	Egg production at the peak (%)	Average monthly losses during production (%)
8.15	8.30	78.6	0.67
12.64	18.01	86.0	0.81
6.71	1.96	85.0	0.43
10.20	8.33	85.3	0.64
11.57	11.31	81.1	0.85
9.52	12.24	75.8	1.02
8.54	3.80	84.0	0.73
5.86	8.73	91.4	1.69
6.41	3.04	85.0	0.62

Table 2

IMMUNOLOGICAL STATUS OF BIRDS

Poultry farm	Anticoccidial preparations used during the rearing period	Percentage of examined sera containing antibodies against						
		IBDV	IBV	ILTV	Adeno-virus	Reo-virus	M. galli-septicum	MDV
I-"KR"	Avatec/Amprol Plus	80.0	0	0	15.0	15.0	100.0	0
II-"SP"	Coyden/Amprol Plus	0	0	0	0	0	100.0	0
III--"DJ"	Cycostat/Amprol Plus	15.0	0	0	0	0	15.0	0
IV-"PW"	Elancoban/Amprol Plus	30.0	0	0	0	0	30.0	0
V-"GJ"	Lerbek/Amprol Plus	100.0	0	0	0	0	100.0	0
VI-"SJ"	Amprol Plus - control	0	0	0	30.0	30.0	100.0	0
VII--"MJ"	Amprol Plus - control	0	0	0	0	0	0	0
VIII--RSP"J"	Amprol Plus - control	100.0	0	0	0	0	100.0	0
IX-"DJ"	Amprol Plus - control	0	0	0	0	0	0	0

The applied anticoccidials fully protected the birds against coccidiosis. The parasitological examinations made in the 4th, 8th, 12th and 20th week of rearing proved to be negative. Similarly, examination of the litter for oocysts of Eimeria spp., performed in the 4th week of rearing, gave a negative result. In the subsequent tests performed in the 8th, 12th and 20th weeks, 50 to 100 oocysts per 1 g of the litter were found. Only on farm No. VI was the number of oocysts higher (in the 12th and 20th week), i.e. 450 and 150, respectively. In the 24th week of the birds' life, the number of oocysts in 1 g of litter increased on the following farms: No. I - 600, No. II - 250, No. IV - 200, No. VI - 300, No. VII - 900.

Tables 3 and 4 present the results of control infection with Eimeria spp. in the 24th week. In the 9 groups infected experimentally with Eimeria spp., only one bird died on one of

Table 3

INFLUENCE OF EXPERIMENTAL INFECTION WITH EIMERIA SPP. ON BODY WEIGHT, MORTALITY AND FEED CONSUMPTION OF CHICKENS

Poultry farm	Anticoccidial preparations used during the rearing period	Average body weight (g)		Survival (%)	Average daily feed consumption during the experiment	
		before infection	on the 8th day		g	%*
I-"KR"	Avatec/Amprol Plus	1740	1711 (-1.67%)	100.0	109	-8.5
II-"SP"	Coyden/Amprol Plus	2649	2445 (-7.70%)	100.0	141	-25.0
III--"DJ"	Cycostat/ Amprol Plus	1433	1518 (+5.93%)	100.0	113	-5.1
IV-"PW"	Elancoban/ Amprol Plus	2670	2816 (+5.47 %)	100.0	194	-3.5
V-"GJ"	Lerbek/ Amprol Plus	2434	2614 (+7.39%)	100.0	200	-0.5
VI-"SJ"	Amprol Plus - control	2405	2562 (+6.53%)	100.0	175	-13.0
VII--"MJ"	Amprol Plus - control	2740	2767 (+0.98%)	100.0	194	-3.5
VIII--RSP"J"	Amprol Plus - control	1570	1522 (-3.06%)	87.5	110	-4.5
IX-"DJ"	Amprol Plus - control	1693	1676 (-1.00%)	100.0	111	-6.7

Explanation: *refers to the period before infection with Eimeria spp.

the farms, i.e. farm No. VIII which served as control. A decrease in body weight occurred in birds receiving Avatec/Amprol Plus on farm No. I (1.7 %), Coyden 25/Amprol Plus on farm No. II (7.7 %), Amprol Plus in a continuous programme on farm No. VIII (3.1 %) and farm No. IX (1.0 %). In all the groups, feed consumption showed a tendency to decrease, especially in birds fed Coyden 25/Amprol Plus (farm No. II; 25 %), Amprol Plus in a continuous programme (farm No. VI; 13 %), and Avatec/Amprol Plus (farm No. I; 8.5 %). Pathological changes were expressed in 2 control groups fed Amprol Plus on farms No. VII and VIII and in birds fed Coyden-25/Amprol Plus on farm No. II. Increased production of oocysts was highly correlated with the presence of pathological changes.

Table 4

LESION SCORE AND OOCYST PRODUCTION IN CHICKENS EXPERIMENTALLY INFECTED WITH EIMERIA SPP.

Poultry farm	Anticoccidial preparations used during the rearing period	Mean lesion score				Mean oocyst production per bird (mil.)
		duodenum	jejunum	caeca	total	
I-"KR"	Avatec/ Amprol Plus	0.28	0.14	0.14	0.56	1.88
II-"SP"	Coyden/ Amprol Plus	0	0.32	1.14	1.46	5.49
III-"DJ"	Cycostat/ Amprol Plus	0	0	0.28	0.28	2.69
IV-"PW"	Elancoban/ Amprol Plus	0.25	0	0.50	0.75	1.18
V-"GJ"	Lerbek/ Amprol Plus	0	0	0.25	0.25	1.92
VI-"SJ"	Amprol Plus - control	0	0.42	0	0.42	3.56
VII--"MJ"	Amprol Plus - control	0	0.33	0.83	1.16	12.09
VIII--RSP"J"	Amprol Plus - control	1.14	0.43	1.71	3.28	18.30
IX-"DJ"	Amprol Plus - control	0	0	0.28	0.28	1.94

DISCUSSION

Of the anticoccidials used in shuttle programme in the present study (Avatec, Coyden-25, Cycostat, Elancoban, Lerbek), only Avatec and Lerbek have been licensed by the E. C. Commission to be applied in chickens reared for layers. According to Weber and Frigg (1984) and Sasmal and Sinha (1984), Avatec used in chickens even in a dose of 90 to 125 ppm enables the development of immunity against coccidiosis. Our observations made under field conditions speak in advocacy of administering lower doses, i.e. 75 ppm of the preparation. The results of our studies concerning the effectiveness of Cycostat in the chemoprophylaxis of coccidiosis in chickens reared for layers are in line with the findings of Karlsson and Reid (1978). According to them, Cycostat in a dose of 33 ppm active compound exerts no suppressing effect at all on the development of immunity against coccidiosis in birds. In birds receiving 90 ppm Elancoban interchangeably with Amprol Plus, mild lesion scores in the alimentary tract and a relatively low production of oocysts were revealed during the challenge. Reid et al. (1977) obtained full immunity against coccidiosis in the 20th week of life by administering 100 to 121 ppm Elancoban during the first 8 weeks of rearing and 50 % of this dose during the subsequent 4 weeks. Among the preparations evaluated, the lowest immunity rate was achieved by applying Coyden-25 interchangeably with Amprol Plus. According to Siegman and Hoeck (1969), this preparation can be used in chickens reared for layers to enable the development of immunity against coccidiosis, but the preparation is recommended to be administered for 3 subsequent days beginning in the 72nd-96th hour after withdrawing Coyden--25 and administering a 0.1 % aqueous solution of sulphamethazine.

On the chicken farms examined, detectable amounts of oocysts in the litter were found in the 8th week of rearing. This might have resulted from a good efficacy of the preparation applied at that time, proper zoohygienic conditions in the chicken houses as well as species of Eimeria occurring in

the environment. According to Braunius (1983), in a later period of rearing (weeks 5-7) peak oocyst score is observed in the birds infected with E. maxima. In the light of the studies of Long et al. (1980), the immunity developed by birds receiving Avatec, Cycostat, Elancoban and Lerbek interchangeably with Amprol Plus in our experiment can be classified to be of degree II-III.

It can be assumed that infection by IBDV, as revealed by the serological tests, did not influence immunity. IBD virus will exert a particularly negative influence on the immune responsiveness of birds if infection takes place in the first days of their life (Radoń, 1980).

Analysing the birds' immunity against coccidiosis, achieved in our study, it can be concluded that more favourable results would be obtained if the dose of Amprol Plus was gradually decreased in the second period of rearing. The differentiated results obtained for the control groups receiving Amprol Plus in a continuous programme can support this assumption. The relatively good immunity achieved on farms No. VI and IX, in comparison with farms No. VII and VIII, can be explained by a decreased sensitivity of Eimeria spp. occurring on these farms to Amprol Plus.

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IMMUNOCHEMOPROPHYLAXIS OF COCCIDIOSIS IN BIRDS

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The difficulties of coccidiosis control depend on some biological features of the parasite. Several immunologically different coccidium species can parasitize the domestic fowl. The immunity against coccidiosis has some peculiarities. Its duration is short and the protection conferred by a single immunization under conditions without reinfection disappears within 50 to 60 days. The degree of immunity is relatively low, rendering possible for some parasites to develop in the organism of immune birds after reinfection. In the field, reinfection of immune birds takes place constantly and results in resistance to coccidiosis.

At present the main method of coccidiosis control is the use of different anticoccidial drugs which can be divided into two groups by their influence on immunity induction. Some of them inhibit the development of immunity to coccidiosis; therefore, they are used only in broilers during the whole rearing period. Other drugs do not prevent the development of immunity and are used in laying and breeder flocks. This is the case when on an anticoccidial background birds develop the immunity protecting them against challenge after withdrawal of the drug. Under field conditions, birds develop immunity asynchronously, and there is a prolonged period during which it is necessary to use anticoccidials. To reduce the time of immunity development in flocks and to decrease the use of chemical drugs, in countries with industrial poultry production studies are focused on the development of immuno-

prophylaxis of coccidiosis. In the United States of America the method for immunization against coccidiosis is patented and on its basis a commercial vaccine, Cocci-Vac, is produced.

The All-Union Veterinary Research Institute of Poultry Science has developed a method for immunochemoprophylaxis of chicken coccidiosis in broilers, breeder and egg-laying flocks (Krylov et al., 1976; Krylova et al., 1982). The method is based on the ability of birds to develop immunity to coccidiosis on a background of drugs not sterilizing the chicken organism from endogenous stages of the parasite. Immunization is carried out by giving in the feed immunogenic oocyst doses of three coccidium species (Eimeria tenella, E. acervulina, E. maxima) not causing clinical signs, and a drug which does not prevent the development of immunity. Birds are immunized once at 10 to 12 days of age; the anticoccidial drug is given in the feed over a period of 20 days, immediately after immunization.

The coccidium strains used for immunization are susceptible to all available anticoccidials and are not contaminated with infectious agents.

This immunoprophylaxis of coccidiosis creates unfavourable conditions for a great deal of oocysts in farms and does not result in outbreaks of the disease (Kindras et al., 1982).

Field trials have shown that this method renders possible a practically complete prevention of morbidity and mortality of birds, an additional weight gain, an improvement of poultry products, an increase of egg production, and a decrease of the duration of use and in the number of anticoccidial drugs.

Furthermore, this immunochemoprophylaxis can be used in practically all poultry farms, even in those where, due to the introduction of anticoccidial drugs, resistant strains of coccidia have appeared.

SUMMARY

Immunization of chickens against coccidiosis is carried out by giving in the feed immunogenic oocyst doses of three coc-

cidium species (Eimeria tenella, E. acervulina, E. maxima) not causing clinical signs, and a drug which does not prevent the development of immunity to coccidiosis. Field trials have shown that this method renders possible a practically complete prevention of morbidity and mortality of birds, an additional weight gain, an improvement of poultry products, an increase of egg production, and a decrease in the duration of use and in the number of anticoccidial drugs.

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COCCIDIOSIS OF FUR-BEARING ANIMALS

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During the spring and summer period we examined coypu, silver fox, arctic fox, mink and sable in collective farms of the Kazakhstan, Altaian and Novosibirsk regions. The test materials were faecal samples bathed in 2 % potassium dichromate solution. Processing of materials was carried out according to the method of Darling. Four species of coccidia were found in coypu: Eimeria pellucida, E. nutriae, E. coypi, Isospora sp.; five species in silver fox: I. buriatica, I. canivelocis, I. vulpina, I. pavlodarica, I. triffitti. The same species of coccidia, together with E. imantaica, were revealed in arctic fox. The coccidia found in mink included E. vison, E. furonis, I. laidlawi and I. eversmanni, while those demonstrated in sable were E. sibirica, E. sablii and I. martesii. Infection rate is high in farms where feeding and keeping conditions fail to meet the animal health and hygienic requirements.

Fur animals of all ages are subject to coccidian infection. Young animals are most susceptible to infection at the age of 2 to 3 months, while 1 month old and adult animals are less susceptible. Clinical coccidiosis occurs among the animals at the age of 2 to 3 months. Our investigations, carried out with experimentally infected animals, showed that coccidiosis might be acute, chronic or latent. The period of incubation lasts 3 to 12 days.

Animals affected with acute coccidiosis show depressed appetite, debility, and marked anaemia of the mucous membranes.

The watery faeces contains large quantities of mucus and, sometimes, detached parts of the intestinal mucosa. The animals exhibit progressive weakness, are reluctant to move and, for the most part, they are recumbent. Prior to their death, disorders of the nervous system (paralysis of muscles in the region of neck and hindlimbs) develop. Most frequently this disease form is observed in young animals after weaning, when they are placed on another feeding regime. The symptoms of chronic coccidiosis are less marked. Although the animals have good appetite, they become retarded in growth and development. As a rule, the latent course is observed in adult and, rarely, in young animals; in that case clinical symptoms are absent.

Coccidian infection of fur animals has a clearly expressed seasonality: it is maximum in the summer and minimum in the winter. Young and adult infected animals are the source of infection as coccidium-carriers. Animals contract infection via the feed and water, into which coccidia may be brought mechanically (by flies or by the operating staff through the things of care).

For some years we have used 15 anticoccidial drugs for treating animals under laboratory and field conditions. These include furazolidone, furacilline, sulfadimezine, norsulfazol Na, biovit, zoalen, coccidin, deccox, iramin, coccidiovit, rigecoccin, clopidol, farmcoccid, coyden-25, and chimcoccid. First, we determined the dose of each preparation, using only a few animals. Subsequently, we tested the drugs on large numbers of animals. The criteria used in the evaluation of efficacy included survival of the animals, clinical symptoms of disease, extensity and intensity of invasion. The following preparations have proved most effective for treating coccidiosis of coypu: farmcoccid in a dose of 0.03, furazolidone: 0.012, zoalen: 0.015, coccidiovit: 0.1, chimcoccid-6: 0.06, coccidin: 0.02, and rigecoccin: 0.01 % in the feed. For treating the coccidiosis of mink the following drugs have proved the best: furazolidone in a dose of 0.02 g/kg body mass or 0.0125 % in the feed; sulfadimezine: 300 mg/kg body mass; chimcoccid: 30 mg/kg body mass or chim-

coccid-6 in a dose of 0.06 % in the feed; coccidiovit: aqueous solution (1 g in 1 litre of water) or 0.1 % in the feed; clopidol: 30 mg/kg body mass or clopidol-25: 0.05 % in the feed; rigecoccin: 40 mg/kg body mass or 0.05 % in the feed; coccidin: 50 mg/kg body mass or 0.02 % in the feed.
In the treatment of silver fox and sable positive results have been obtained with furazolidone given in a dose of 0.012 %, sulfadimezine applied in a dose of 0.02 %, and chimcoccid-6 used at a dose rate of 0.06 % in the feed. The therapy of fox and sable coccidiosis requires further investigations. It must be noted that sulfadimezin is given to animals over two 3-day periods with an interval of 2 days. Prolonged application of this preparation is contraindicated. Chimcoccid is effective when given over two 5-day periods with an interval of 3 days. The latter preparation must be given to animals over a period of 7 to 8 days. If necessary, the period of treatment may be prolonged or repeated after a certain interval.

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EFFECT OF LASALOCID AGAINST COCCIDIOSIS AND ON WEIGHT GAIN IN LAMBS

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In South Poland, coccidiosis is an important disease which affects the profitability of sheep breeding. The economic consequences of coccidial infections are especially remarkable in lambs. It is worthy to note that in Poland we do not have a control program and only a symptomatic treatment of lambs is used.

In the last years there have been several reports of effective coccidiosis control in lambs using the polyether antibiotics monensin sodium (Fitzgerald and Mansfield, 1978; Horton and Stockdale, 1979, in press; McDougald and Dunn, 1978), salinomycin (Serwin, 1983; Schlolaut et al., 1983), and lasalocid (Foreyt et al., 1979; Horton and Stockdale, 1981; Jensen, 1974). These drugs are not only effective anticoccidials but they also improve ruminal VFA patterns, efficiency of feed conversion and average daily weight gain.

The present study was carried out to observe the influence of feeding lasalocid on coccidiosis and weight gain in lambs.

MATERIALS AND METHODS

Coccidial infection was monitored in three different types of farms, i.e. state, collective, and private. In the years 1982 through 1983 a total of 3650 faecal samples were examined. One hundred and twenty lambs (mean body weight: 16.5 kg) of the Polish Mountain breed were used to study the influence of lasalocid on coccidiosis and weight gain. The lambs were ran-

domly allotted to two groups with three pens (replications) of 20 lambs per group. The first group included nonmedicated, naturally infected control animals, the second one naturally infected lambs fed 20 mg lasalocid/kg of diet. The medicated feed was given to the lambs continuously from 15 May to 30 October, i.e. over a period of 170 days. At 14-day intervals the lambs were weighed and faecal samples were collected for parasitological examination. Evaluation was made according to the method of Willis and Schlaaf.

RESULTS

In Table 1 the extensity of coccidial infection in sheep from three types of farms (state, collective, and private) is presented. The extensity of infection among lambs of collective, state, and private farms was 79.6, 54.4 and 24.4 %, respectively. Cases of acute clinical coccidiosis occurred in the experimental farms.

Table 1

EXTENSITY OF COCCIDIAL INFECTION IN SHEEP IN THREE TYPES OF FARMS (STATE, COLLECTIVE AND PRIVATE)

Type of farm	Number of animals	Number of infected animals	%
State	2400	1306	54.4
Collective	960	765	79.6
Private	290	71	24.4
Total	3650	2142	58.6

The following Eimeria species were found in sheep from the mountain region: E. pallida, E. parva, E. nina-kohl-yakimovi, E. faurei, E. intricata and E. arloingi. E. parva (36.1 %), E. nina-kohl-yakimovi (33.5 %) and E. faurei (30.2 %) were the most frequently found species.

In Figure 1 the seasonal dynamics of coccidiosis in treated

and nontreated lambs is followed. In lambs, the first infections were observed in March. In nontreated animals the highest extensity and intensity were found in the period from July to October. In this period about 85 to 90 % of the animals were infected. In the control group the first deaths due to coccidial infection were established in May and June. Among the animals treated with lasalocid no clinical coccidiosis and mortality were observed. The extensity of infection was very low and single oocysts were found in only 5 to 10 % of the treated lambs (Fig. 1).
Table 2 presents the influence of lasalocid on weight gain and wool production. The final weight was higher by about 5.2 kg in the lasalocid-treated group than in the control, nontreated group. Wool production was higher by 320 g in the lasalocid-treated group than in the control one.

DISCUSSION

Faecal examinations showed that in lambs the highest extensity and intensity of coccidiosis occurred in the period from July to October. E. parva, E. nina-kohl-yakimovi and E. faurei were the most frequently found species. These observations are in agreement with results of other Polish authors (Fagasiński, 1973; Kozakiewicz, 1981; Patyk, 1964; Ramisz et al., 1971; Serwin, 1983). A similar situation was found by Jungmann et al. (1973) and Neetzow et al. (1966) in the G. D. R., where the most severe coccidial infections of lambs were observed in the period from June to October.
Lasalocid in a dose of 20 mg/kg of diet effectively controls ovine coccidiosis. In the treated lambs the extensity of infection was very low and single oocysts were found in only 5 to 10 % of the animals. Similar observations were made by Jensen (1974), Foreyt et al. (1979) and Horton and Stockdale (1981). Jensen (1974) reported that both monensin and lasalocid in a dose of 22 and 100 mg/kg of diet, respectively, eliminated E. nina-kohl-yakimovi and E. ahsata, the two major pathogenic species known to affect sheep in North America. Foreyt et al. (1979) reported an effective control of patho-

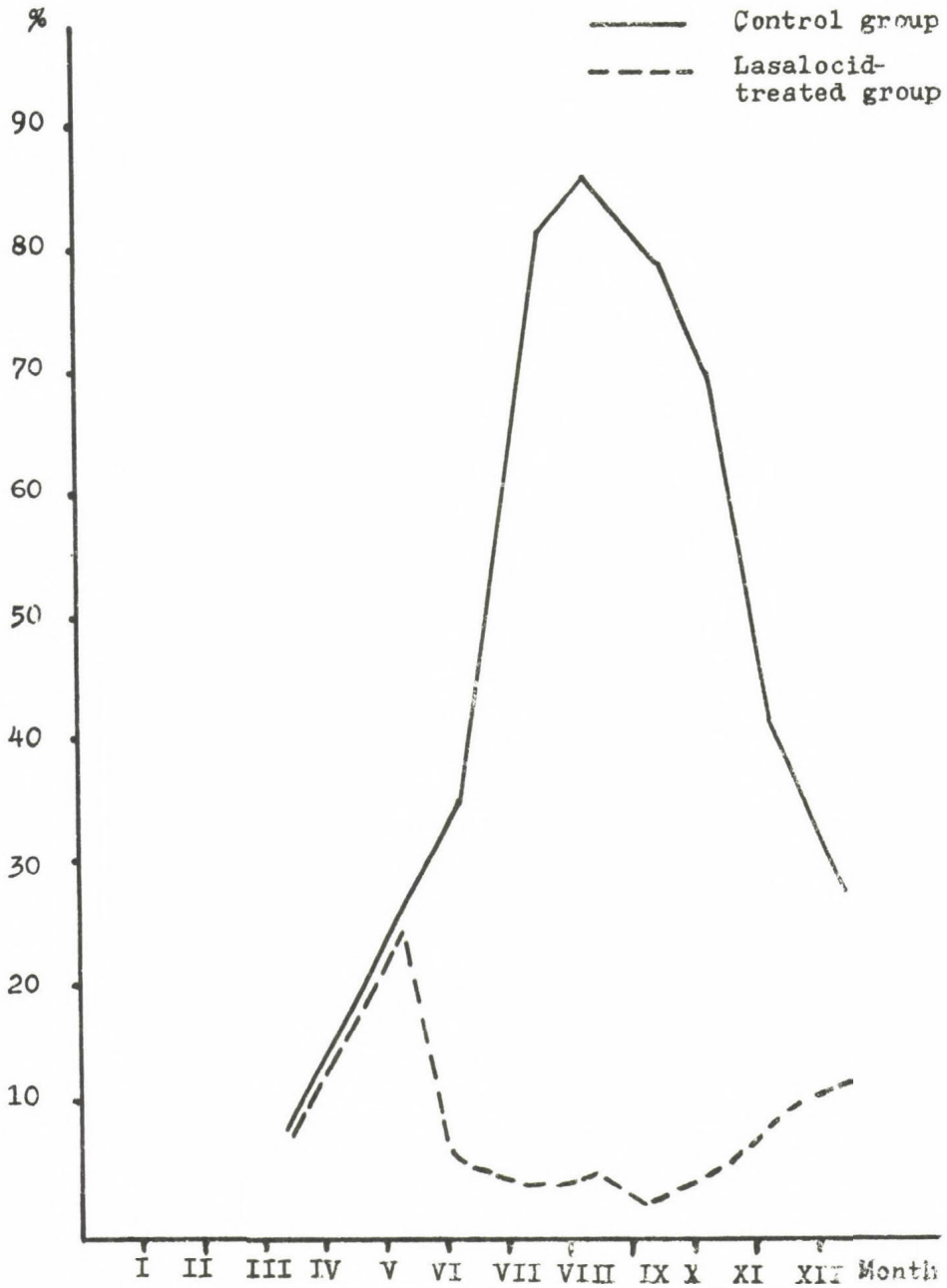

Figure 1. Seasonal dynamics of coccidiosis in lambs treated and not treated with lasalocid

genic ovine coccidia with lasalocid and monensin 28 days after inoculation with Eimeria spp. Horton and Stockdale (1981) fed 12.5, 25, 50 and 100 mg lasalocid/kg of diet and established the optimal dose between 25 and 50 mg/kg of diet, as indicated by oocyst output, feed efficiency, daily gains and ruminal fermentation patterns.

In lambs fed lasalocid an improved weight gain was observed. The final weight of lasalocid-treated lambs was about 5.2 kg higher than that of the control, nontreated animals. The trend to higher weight gain and better feed conversion in lasalocid-treated lambs is in agreement with the results of Foreyt et al. (1979) and Horton and Stockdale (1981).

It is of interest that in the lasalocid-treated group wool production was also higher than in the control one.

Table 2

WEIGHT GAIN AND WOOL PRODUCTION OF LAMBS NATURALLY INFECTED WITH EIMERIA SPP. AND TREATED WITH LASALOCID

Parameter	Experimental group (20 mg lasalocid/kg of diet)	Control group	Difference (kg)
Initial weight (kg)	16.9	16.7	0.2
Final weight (kg)	46.2	40.8	5.4**
Wool production (kg)	1.92	1.60	0.32**

**Statistically significant difference

The results of this study indicate that lasalocid is an effective therapeutic agent against coccidiosis in lambs. At a dose rate of 20 mg/kg diet, lasalocid reduced oocyst output and improved weight gain as well as feed conversion.

SUMMARY

Coccidial infection was followed in sheep from three types of farms, i.e. state, collective and private. The highest ex-

tensity was found in collective farms where the proportion of infected animals was 79.6 %. Six Eimeria spp. were found in sheep from the mountain region; of them E. parva (36.1 %), E. nina-kohl-yakimovi (33.5 %) and E. faurei (30.2 %) occurred most frequently. The seasonal dynamics of coccidiosis in lambs treated with lasalocid (20 mg/kg of diet) and in nontreated lambs was monitored. In nontreated animals the highest extensity and intensity were found in the period from July to October (85 to 90 % infected lambs). In lambs treated with lasalocid the extensity was very low and single oocysts were found in only 5 to 10 % of the treated animals. The final weight was higher by about 5.2 kg and wool production by about 0.32 kg in the lasalocid-treated group than in the control, nontreated group.

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PROGRAM OF ROTATION OF MONENSIN AND LASALOCID IN THE ČSSR

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Monensin has been used in the Czechoslovak broiler industry since 1978. In 1981, i.e. in the 4th year of its use, 8 field isolates of Eimeria tenella were obtained from farms suffering from acute outbreaks of coccidiosis (Bedrník, 1983; Bedrník et al., 1985). These isolates were repeatedly tested for sensitivity to monensin and lasalocid in floor-pen trials.

MATERIALS AND METHODS

From the 1st day of their life the experimental Ross I sexed cockerels were kept in an environment-controlled hall. They were fed a broiler starter ration supplemented with 60 ppm of arprinocid. The trials started when the chickens were 21 days old. Groups of 30 birds with the same weight distribution and with statistically uniform average liveweight were formed. Each group was placed in a separate pen. From that time on, the chickens were fed a broiler grower feed supplemented with the drugs to be tested. When 25 days old, the chickens were infected via the feed with 25×10^3 oocysts of one of the isolates. Chickens in four pens were infected with each respective isolate: birds in one of these four pens were treated with monensin (100 ppm), those in the other one with lasalocid (75 ppm), and chickens in the third one with arprinocid (60 ppm). Chickens of the fourth pen received no treatment. The trials were terminated when the chickens reached an age of 49 days. The main parameters followed were

body mass gain, feed conversion, and mortality caused by coccidia.

RESULTS

The main results are summarized in Table 1.

Table 1

SENSITIVITY OF FIELD ISOLATES OF EIMERIA TENELLA TO MONENSIN AND LASALOCID IN 1981 AND 1984

Isolate		Isolated in 1981		Isolated in 1984	
		MON.	LAS.	MON.	LAS.
1	Březina	+	+	nd	
2	Příšovice	+	+	±	+
3	Velká	+	+	+	+
4	Jelence	±	±	nd	
5	Xaverov	±	+	±	+/±
6	Bynina	±	+	no coccidia	
7	Jesenice	+	+	nd	
8	Pičín	±	+	no coccidia	

Monensin: 100 ppm; lasalocid: 75 ppm

Four of the eight isolates tested proved to be less sensitive to monensin. The control of these isolates with lasalocid was in 3 cases better than with monensin, while in one case it was of the same degree. The reason for this decreased sensitivity was permanent underdosage of monensin in the feed used on the respective farms.

The incidence of coccidiosis in broiler production decreased after the introduction of lasalocid (90 ppm) in Czechoslovakia in 1982. It alternates with monensin on a district basis in 12-month periods. On the other hand, problems with wet litter occur on some farms during winter periods due to the high salt content of the diet.

In 1984 we tried to reisolate coccidia from the same farms as in 1981 and evaluate their sensitivity to monensin and

lasalocid. According to the results presented in Table 1 we can say that while on farms with a good management problems due to coccidiosis have disappeared, on the farms with a low zoohygienic standard and a continuing underdosage of anticoccidial drugs in the feed the local strains of coccidia are not only less sensitive to monensin but demonstrate the first signs of decreasing sensitivity to lasalocid as well.
In addition to the above-listed isolates, in the period 1983 through 1985 we obtained other three field isolates which demonstrate some signs of decreased sensitivity to monensin or lasalocid (see Table 2).

Table 2

SENSITIVITY OF FIELD ISOLATES OF E. TENELLA (1983-1985) TO MONENSIN AND LASALOCID

Isolate	Year of isolation	Sensitivity to MON.	Sensitivity to LAS.
1 Choltice	1983	±	+
2 Rozhovice	1985	±	+
3 Miřetice	1985	+	±

Monensin: 100 ppm; lasalocid: 75 ppm

In the case of lasalocid, however, the reduced sensitivity concerns the dose 75 ppm, which was used in our trials, while in broiler production a dose of 90 ppm is applied. Sensitivity to 90 ppm needs to be verified.
In order to preserve the activity of ionophore anticoccidial drugs a shuttle program has been used since early 1985. In broiler starter diets snythetic anticoccidial drugs will be used. They will be changed regularly after one year of use. In broiler grower diets monensin and lasalocid will alternate as up to now, i.e. on a district basis.

SUMMARY

Rotation of monensin and lasalocid in 12-month periods result-

ed in a better control of coccidiosis than when only monensin had been used.

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CONTRIBUTION TO OUR KNOWLEDGE ON THE MODE OF ACTION OF MONENSIN

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In 1981 Smith et al. revealed the actual mode of action of monensin against coccidia. The penetration of extracellular Eimeria tenella sporozoites into cultured cells substantially decreased after a 4-h treatment with monensin. However, this period of exposure to monensin is nonphysiologically long because coccidian sporozoites and merozoites penetrate the cells very quickly. Fayer and Hammond (1967) detected intracellular sporozoites 3 min, and Bedrník (1969) found intracellular merozoites 30 sec, after inoculation into a cell culture. Therefore, we followed the activity of monensin against E. tenella sporozoites during physiological periods of excystation and penetration into cultured cells.

MATERIALS AND METHODS

Freshly excysted sporozoites were incubated in a 0.1 μg/ml solution of monensin in phosphate buffered saline (PBS) for 2, 4, or 8 min, respectively. The action of monensin was interrupted by dilution with cold PBS, and monensin was removed by repeated centrifugation. Sporozoites resuspended in a tissue culture medium based on lactalbumin hydrolysate supplemented with 10 % of calf serum were inoculated into cell cultures derived from chicken embryos. After 1 h of cultivation at 41 °C the slides with cell monolayer were fixed and stained with haematoxylin and eosin. Intracellular sporozoites were counted on 4 slides, in 25 visual fields on each

slide, using an Orthoplan microscope with an immersion objective of 100x magnification.

RESULTS

As we can see from Table 1, the number of intracellular sporozoites was substantially reduced even after a 2-min exposure to monensin.

Table 1

MONENSIN TREATMENT OF EXTRACELLULAR SPOROZOITES
Influence on their penetration in cell cultures

Time of monensin (0.1 μg/ml) action (min)	Number of intracellular sporozoites 1 h post-inoculation; trial no.			
	1		2	
	pcs	%	pcs	%
0	133	100	35	100
2	85	64	14	42
4	99	74	11	33
8	95	71	12	36

Inoculum: 2×10^5 sporozoites of E. tenella per tube

In other trials the further development of sporozoites treated with monensin as mentioned above was followed. After 4 days of cultivation the number of schizonts of the 1st generation was remarkably reduced, in comparison with the control cultures (see Table 2).

Table 2

MONENSIN TREATMENT OF EXTRACELLULAR SPOROZOITES
Influence on their further development

Time of monensin (0.1 μg/ml) action (min)	Number of the 1st and 2nd schizonts on the 4th day post-inoculation	
	pcs	%
0	68	100
2	16	25
4	15	24
8	17	26

Inoculum: 2×10^5 sporozoites of E. tenella per tube

In the subsequent trials the influence of monensin on the course of excystation of oocysts was observed. The concentration of monensin in the excystation solution of trypsin and bile (Jackson, 1964) was again 0.1 μg/ml. Monensin had no negative effect on the number of excysted sporozoites; however, their viability was again remarkably reduced (see Tables 3 and 4).

Table 3

MONENSIN ACTION DURING EXCYSTATION

Time of excystation (min)	Number of excysted sporozoites (x 10^4)	
	in presence of monensin	in absence of monensin
15	19	22
30	48	40
45	60	61
60	63	77

Table 4

MONENSIN ACTION DURING EXCYSTATION

Time of excystation (min)	PENETRATION OF EXCYSTED SPOROZOITES INTO CULTURED CELLS No. of excysted sporozoites 1 h p. i.			
	excysted + MONENSIN		excysted-MONENSIN	
	pcs	%	pcs	%
15	6	32	18	100
30	6	33	19	100
45	5	25	18	100
60	5	23	21	100

Inoculum: 1.5 x 10^5 sporozoites per tube

Finally, the influence of monensin on intact sporocysts was monitored. A 24-h exposure of sporocysts to monensin at 41 °C did not affect the course of excystation.

It can be concluded that exposure of E. tenella sporozoites to 0.1 µg/ml monensin for 2 min results in a remarkable reduction of sporozoite viability. Monensin has no effect on the viability of sporozoites inside the intact sporocysts.

SUMMARY

E. tenella sporozoites were treated with 0.1 µg of monensin per ml for 2 min. Subsequently, their penetration into cells was up to 3 times lower. Also the further development of penetrated sporozoites was lower than that of the untreated sporozoites. The presence of monensin during excystation did not influence the number of released sporozoites. The viability of sporozoites excysted from sporocysts treated previously with monensin remained unaltered.

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THE USE OF TOLTRAZURIL-MEDICATED FOOD TO PREVENT THE DEVELOPMENT OF ISOSPORA AND TOXOPLASMA OOCYSTS IN DOGS AND CATS

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Intrauterinely acquired Toxoplasma infections frequently result in serious disease of the new-born child followed by death or lasting damages of the central nervous system. According to American reports (Wilson, Remington 1980) 1.1 o/oo of all children are born with a congenitally acquired Toxoplasma infection. The annual costs for therapy and maintenance of these children in the United States are estimated at 222 million dollars. Only children of women who acquired their first infection during pregnancy are at risk. Man may contract the infection in his postnatal life from two sources: The oral uptake of raw or undercooked meat containing Toxoplasma cysts or through oocysts from the faeces of cats.

The prevention of infection by cysts is relatively easy: Pregnant women should not consume raw or undercooked meat and they should wash their hands thoroughly after the handling of meat. The prevention of infection by oocysts is a bigger problem, especially when cats are kept in the household. Oocysts are very resistant to environmental conditions and may survive for at least one year on the ground. Cats usually excrete oocysts in large numbers only once in their life for about one week. However, the following immunity is not very strong, and some of the cats may again excrete oocysts after reinfection. Even without reinfection, reshedding of small numbers of oocysts may occur. At present, the only advice which can be given to seronegative women planning to have a baby is to give away their cat and to avoid contact with other cats or areas where cats may de-

faecate. We therefore have investigated the possibility to prevent oocyst shedding by medicating the food of cats with an anticoccidial drug (Schnieder 1982, Westerhoff 1984, Krause 1986).

With the exception of monensin, none of the substances so far tested by other authors (sulphonamides, potentiated sulphonamides, clindamycin) were able to suppress the excretion of Toxoplasma oocysts completely. Very good results were obtained with monensin (Frenkel, Smith 1982). However, this substance is very toxic, even when only slightly overdosed. Since excellent results have been reported using toltrazuril (Bay Vi 9142, Bayer AG) against coccidial infections in mammals (Haberkorn, Schulz 1981, Haberkorn, Stoltefuss 1984), the ability of this drug to prevent the excretion of Isospora and Toxoplasma oocysts in dogs and cats was investigated.

In a preliminary trial, each of ten coccidia free beagles was infected with 100 000 sporulated oocysts of Isospora burrowsi. Five dogs were treated with 10 mg per kg toltrazuril daily from day 2 to 6 p.i. The drug was mixed into the food and was taken up readily. Five dogs remained untreated. None of the treated dogs excreted oocysts, whereas all control dogs shed between 7.5 and 37 million oocysts during a patency of 12 to 16 days and after a prepatency of 7 days. Following reinfection of the treated animals on day 40 after the first infection, 4 dogs proved to be immune and one dog excreted 3.5 million oocysts after a prepatency of 7 days during a patency of 12 days .

Encouraged by these results, we started some experiments to determine the efficacy of toltrazuril on the sexual stages of Toxoplasma gondii in the intestinal wall of the cat. In a first trial each of 3 seronegative cats in 7 groups were infected once by oral application of 2 latently infected mice (Toxoplasma strain of pig origin). Two untreated groups were used as positive controls, three groups were treated once with 10, 20 or 40 mg per kg toltrazuril on day 2 p.i., one group was treated once with 20 mg per kg on day 3 p.i. and one group with 10 mg per kg daily from day 1 to 5 p.i. Another 5 cats in two

groups of 3 and 2 animals were infected with 1 or 2 mice on day 0, 2, 4, 6 and 8 of the experiment and treated with 5 or 10 mg per kg toltrazuril daily for 15 days, starting one day before the first infection. All cats took up the medicated food without hesitation. After a prepatency of 3 to 7 days, the 6 cats of the two control groups excreted between 47 and 441 million oocysts during a patency of 10 - 17 days. All but one of the cats of the 4 groups treated only once excreted between 0.06 and 560 million oocysts, whereas the faeces of all 3 cats which were treated with 5 mg per kg daily from day 1 to 5 p.i. remained negative. Moreover, 4 of the 5 cats infected repeatedly and treated daily with 5 or 10 mg per kg toltrazuril remained negative. Only one cat of these two groups which had received 10 mg per kg toltrazuril daily shed 39 million oocysts. This cat had severe diarrhoea of unknown cause before and during the experiment. Twenty-three of the 26 cats of this series were reinfected 42 to 47 days after the first infection and all were immune regardless whether they had excreted oocysts after the first infection or whether they had received toltrazuril or not.

In order to confirm the efficacy of toltrazuril in preventing Toxoplasma oocyst shedding, another 12 seronegative cats were put on a medicated diet (28.25 mg toltrazuril per cat and day). Six cats served as positive control and received normal diet. Six medicated cats and 3 control cats were fed 5 latently infected mice 3 days after the start of medication and 6 medi cated cats and 3 control cats were fed 1 latently infected mouse every 2nd day for 4 months. The 6 cats on normal diet excreted oocysts between day 4 and 8 after the first infection and remained negative thereafter. Also one of the 12 cats on toltrazuril diet excreted 240 000 oocysts on day 6 after the first infection. The 11 other cats on toltrazuril diet remained negative. At the end of the experiment all cats were serologically (IFAT) positive and had cysts in their extraintestinal organs regardless whether they had received medicated food or not.

In the second part of the experiments we examined the ability of toltrazuril to prevent the development of Toxoplasma oocysts

following the migration of parasites from extraintestinal organs to the gut wall, i.e. after the inoculation of oocysts. Thirty-three cats were inoculated orally with 50 to 500 000 sporulated oocysts of T. gondii. Seventeen of them were treated with 5 mg toltrazuril per kg of body weight daily from day 3 p.i. for 63 days. Ten cats of each group were additionally immunosuppressed (0,5 to 2 ml depot-dexamethason - Voren(R), Boehringer Ingelheim - i.m. on day 18, 22 and 26 p.i.). Three out of the 16 cats that were inoculated with oocysts and not medicated with toltrazuril (2 of them immunosuppressed) excreted oocysts for 3 to 7 days after a prepatency of 23 to 33 days. The faeces of all 17 cats medicated with toltrazuril remained negative. Following reinfection with cysts 64 or 88 days after the inoculation of oocysts, all 3 cats that had excreted oocysts after the initial infection were immune. Some of the remaining 29 cats were immune and others excreted oocysts after reinfection with cysts. The reason why some of the cats had become immune and others not remained obscure.

Finally 10 seronegative cats were inoculated with 750 000 oocysts per cat 4 times 3 days apart. Five of them were put on toltrazuril diet throughout the experiment starting 5 days before the first inoculation of oocysts. None of the cats of both groups excreted unsporulated oocysts. Interestingly, all cats on medicated diet remained serologically negative, whereas the 5 control cats became positive.

In order to test whether toltrazuril can also prevent the reshedding of oocysts in the absence of reinfection 40 latently infected seropositive cats were immunosuppressed (1 x 2, 5 x 5 or 2 x 10 ml depot-dexamethason i.m. 4 days apart). Eighteen of these cats were on normal and 22 on toltrazuril diet. Three of the non medicated 18 cats reshed oocysts for 3 to 7 days starting 2 to 14 days after the first injection of dexamethason. The faeces of all cats on toltrazuril diet remained negative.

In conclusion it can be stated that Toxoplasma oocyst shedding and the phenomenon of reshedding can be minimized by medicating

the food of cats with toltrazuril. Oocyst shedding was completely suppressed in cats inoculated with oocysts and in latently infected cats, whereas 2 of 20 cats infected by the oral uptake of cysts excreted a reduced number of oocysts inspite of medication. By using a medicated diet for cats in the environment of pregnant women in addition to the conventional methods of precaution the risk of infection during pregnancy followed by Toxoplasma infected children probably could be reduced significantly.

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EFFICACY OF EsB_3 AGAINST *EIMERIA STIEDAI* IN RABBITS

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ABSTRACT

Groups of 6-week-old New Zealand White rabbits were infected each with approx. 7o,ooo sporulated oocysts of *E.stiedai* on a single occasion or spread over a lo day period. The animals received o.1% Esb_3 in the drinking water either continuously from days -1 to 21 after infection or for a 5 days' period, ie. days -1 to 4, days 5 to lo or days 12 to 17. Uninfected and infected groups were included as controls. All rabbits were killed on day 21 after infection.

Enlarged livers with severe gross lesions and enormous amounts of oocysts in the bile were present in each animal of the infected groups, while complete control of the parasite was observed in the rabbits having received medication continuously or between days 5 to lo after infection.

INTRODUCTION

The gross lesions in bile ducts due to infection with *Eimeria stiedai* are regarded universally as the most common reason for liver condemnation of broiler rabbits in slaughterhouses. Among various anticoccidials, many sulphonamide compounds have long been tested for their efficacy in rabbits, primarily against species of *Eimeria* dwelling in the intestinal tract /see review by Dürr and Schrecke, 197o/.

There is a conspicuous dearth of knowledge on the efficacy of Esb_3 /containing 3o% sulphachlorpyrazine sodium monohydrate and 7o% cane sugar/ on coccidiosis of rabbits. Dürr and Lämmler /197o/ reported disappointing action against intestinal coccidiosis in rabbits while improved condition and reduced oocyst counts were observed by Ndokue /1977/ in naturally infected animals. Our scrutiny of literature yielded a single paper dealing with its action agsinst E.stiedai /Kolabskiy et al., 1973/. In this paper sulphachlorpyrazine given from day 4 after experimental infection at a daily dose of 5o mg kg^{-1} body weight in the feed over a period of lo days proved to be loo% effective. Administration in the drinking water /1 g in 3 liters/ over a period of lo days resulted in 88 to loo% efficacy in rabbits with severe natural infection of E.stiedai.

Since no further information could be obtained from the manufacturer /CIBA-GEIGY/, we decided to test the activity of this drug against experimental infection with E.stiedai. The result of this trial is summarized in the present paper.

MATERIAL AND METHODS

Animals

A total of 8o conventional New Zealand White rabbits, born and reared free of hepatic coccidiosis in the Environmental Laboratory of the Animal Hygiene Department of the Univ. of Vet. Sci. were used in the trial. After weaning, the animals at the age of 42 to 45 days were housed in groups of 5 rabbits per wire-floored metal cage and were kept at 19 to 22 ^{o}C ambient temperature.

Feed

Pelleted feed containing no anticoccidial drug was given ad libitum and the daily consumption was registered. Esb_3 was administered as o.1% solution in the drinking water and the daily consumption in each group of rabbits measured. This enabled us to estimate the mean daily intake of the Esb_3 as

well as of vitamin-K added as a supplement for one group of rabbits.

Infective material

A strain of E.stiedai was isolated from a lethal case of hepatic coccidiosis and, after one passage, harvested from experimentally infected rabbits. Infective doses of sporulated oocysts were inoculated into the oesophagus by means of a curved metal tube attached to a syringe.

Experimental design

Eight groups of 1o rabbits each /excepting sub-groups 4/A and 4/B/ were used for infection or infection plus administration of ESB_3. ESB_3 was added as a o.1% solution in drinking water. The groups were as follows:

Group 1: Uninfected untreated controls /UUC/
Group 2: Infected with 7o,ooo sporulated oocysts of E.stiedai on day 0 /IUC/
Group 3: Infected as IUC, medicated continuously from day - 1 till day 21 after infection /IM -1 to 21/
Sub-group 4/A: 5 rabbits inoculated with daily dose of 7,ooo oocysts from day 0 to day 9 and medicated from day -1 till day 21 after infection /1 FM -1 to 21/
Sub-group 4/B: 5 rabbits inoculated as 4/B but remained untreated controls /IFUC/
Group 5: Infected and treated as group 3, however, given also vit.-K at 3 mg l^{-1} drinking water /IM -1 to 21 K/
Group 6: Infected as IUC, medicated from day -1 till day 4 after infection /IM -1 to 4/
Group 7: Infected as IUC, medicated from day 5 to day 1o after infection /IM 5 to 1o/
Group 8: Infected as IUC, medicated from day 12 to day 17 after infection /IM 12 to 17/.

The mean number of oocysts per gram of faeces /OPG/ in each group of rabbits was determined in a McMaster counting

chamber at regular intervals from day -2 to 2o after infection. The body weight of ear-tagged rabbits was recorded each week. All animals were slaughtered on day 21 after infection, lesion scores on the liver surface /using the semi-quantitative scale of Sambeth and Raether, 198o/ and the presence of oocysts in the bile examined. The liver weight was measured.

RESULTS

Medication of rabbits with administration of Esb_3 in the drinking water at a level of o.1% over periods of 5 to 21 days failed to show any apparent reduction of oocyst output from mild naturally acquired coccidial infection of the intestinal tract by E.magna, E.media and E.perforans. Oocysts output measured o to $59x1o^3$ OPG.

No oocysts of E.stiedai were detected after the prepatent period /17 days/ in the faeces of group IM -1 to 21, sub-group IFM -1 to 21, group IM -1 to 21 K or of group IM 5 to 1o. A few oocysts of E.stiedai were, however, noted in group UUC, while enormous amounts of them were discharged in the rest of groups /and sub-group IFUC/ with $1,61oxlo^3$ OPG in IUC.

It was a characteristic feature of faeces in group IM 12 to 17 that among the few normally shaped oocysts many irregular, indented oocyst walls lacking sporonts appeared /Fig.1/. In the bile of these rabbits, mature oocysts scarcely were seen as the bulk of them showed irregular shape with the sporont filling up almost the whole cavity of the oocyst /Fig. 2/. They were unable to sporulate.

At postmortem, severe hepatic coccidiosis invariably with a lesion score of 4 and large amounts of oocysts in the content of gall bladder were observed in each rabbit of group IUC, sub-group IFUC, groups IM -1 to 4 and IM 12 to 17. Rabbits in the rest of groups and sub-group IFM -1 to 21 showed

Fig. 1. Irregular oocysts of E.stiedai showing indented oocyst wall and lacking sporont

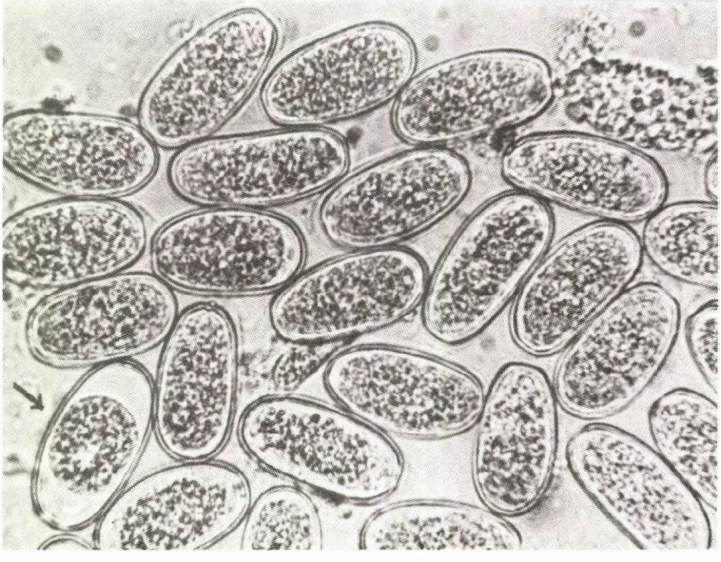

Fig. 2. A single normal (arrow) and immature oocysts of E. stiedai in the bile

neither gross lesions in the liver nor oocysts in the bile. One exception was a single rabbit in UUC that showed 2 to 3 subtle spots on the surface of liver /score:1/ and many mature oocysts in the bile. This was indicative of an incidental infection.

Fig. 3. Normal livers of rabbits in sub-group IFM -1 to 21 /4/A/ and those showing severe lesions of coccidiosis in sub-group IFUC /4/B/

Fig. 3 demonstrates livers of normal appearance and those of severe hepatic coccidiosis in sub-groups IFM -1 to 21 and IFUC, respectively. The mean weight of livers /$\pm$ S.D./ in different groups of rabbits 21 days after infection is shown in Fig.4. Some of the parameters used to evaluate the efficacy of Esb_3 are summarized in Table 1. If the body weight gain in different groups is compared to group 2 instead of group 1, significant /$p<0,05$-0.001/ improvement can be established for all groups except 8 and sub-group 4/B.

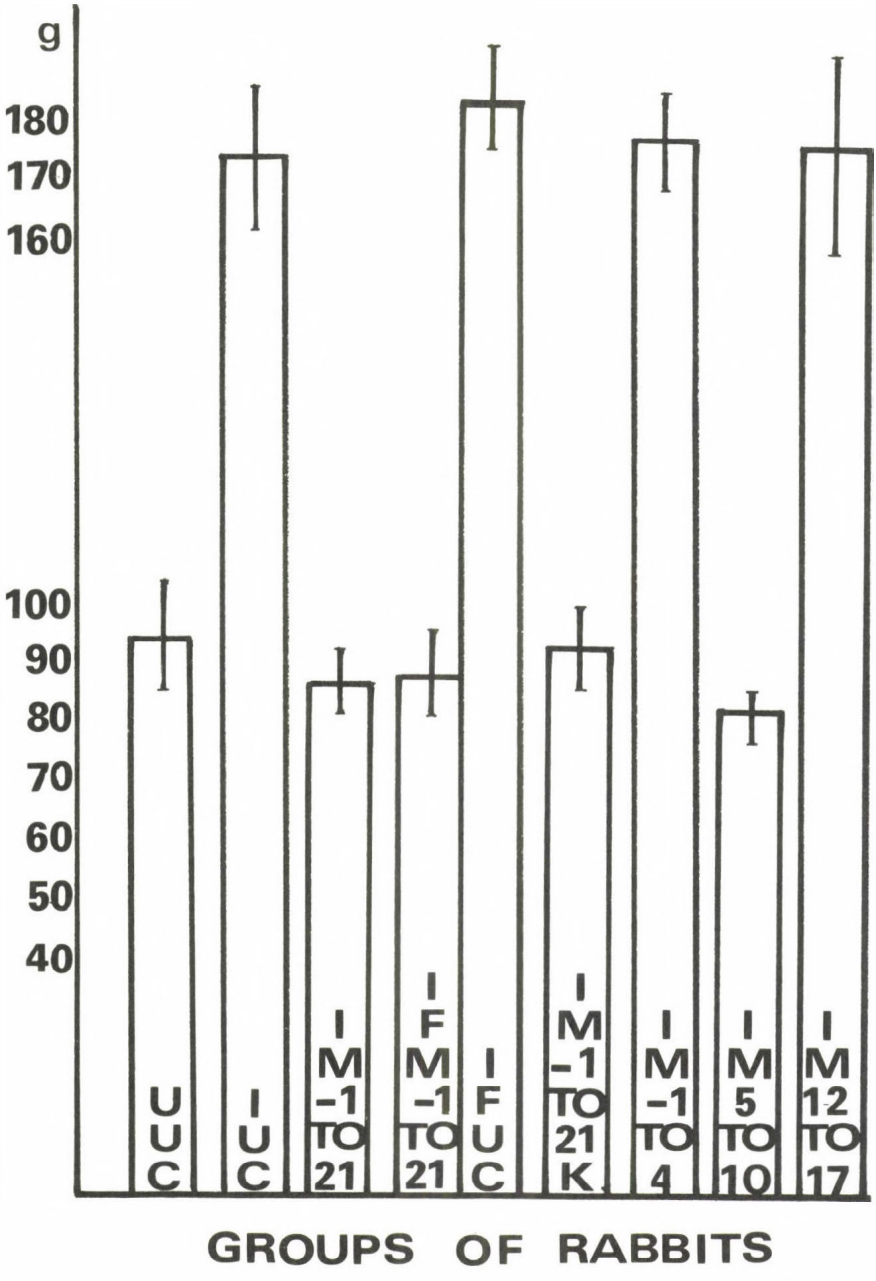

Fig.4. Mean weight of livers (g $\pm$ S.D.) in medicated and control groups of rabbits 21 days after infection with E.stiedai

Table 1. Some relevant parameters used to check the efficacy of Esb_3 against E.stiedai in rabbits

Group	Livers with lesions	Body weight (g) on day -1 $\bar{x} \pm$ S. D.	Bwt. gain excl. liver weight (g) over 22 days $\bar{x} \pm$ S. D.	Bwt. gain excl. liver weight (g) difference from group 1 (P)	Feed conversion ratio (excl. weight of liver)	Esb_3 (g) consumed per rabbit
1	1/10	878.5 ± 52.1	636.8 ± 31.3	-	3.20	-
2	10/10	892.5 ± 29.4	364.7 ± 52.0	< 0.001	4.00	-
3	0/10	936.5 ± 29.9	704.4 ± 27.4	> 0.05	2.96	3.570[x]
4/A	0/5	1145.0 ± 133.2	689.2 ± 62.8	> 0.05	3.13	4.540[x]
4/B	5/5	1018.0 ± 43.2	413.4 ± 46.2	< 0.001	4.42	-
5	0/10	1025.0 ± 33.9	654.4 ± 36.4	> 0.05	3.30	4.347[x]
6	10/10	1101.0 ± 70.5	542.4 ± 57.8	> 0.05	4.14	0.735[xx]
7	0/10	936.0 ± 50.7	609.1 ± 14.6	> 0.05	3.07	0.938[xx]
8	9/9*	944.0 ± 34.9	316.0 ± 80.6	< 0.001	5.01	0.657[xx]

[x] during 21 days; [xx] during 5 days
* 1 rabbit died of enteritis

Neither water intake nor feed consumption was consistently altered by the medication. In group IM -1 to 4, however, anorexia and reduced feed consumption was noted in the final stage of the trial. Vit.-K supplement proved to have no effect.

DISCUSSION

In contrast with Ndokue's /1977/ finding, our failure to control oocyst production of intestinal coccidia in mildly infected rabbits by administration of Esb_3 is rather in line with Dürr and Lammler's /197o/ report.

As for the activity of Esb_3 against liver coccidiosis, results of the present study are in harmony with those of Kobalskiy et al. /1973/ who treated their rabbits over a period of lo days.

In our trial the medication of rabbits fed merely on pelleted rabbit feed and continuously receiving Esb_3 as o.1% solution of drinking water showed a complete efficacy against both severe single inoculation with oocysts of E.stiedai /groups 3 and 5/ and inoculation spread over lo days /sub--group 4/A/. Reduction of drug administration to 5 days resulted in an efficacy closely dependent on the stage of development of the parasite. While complete chemoprophylaxis was achieved by treatment between days 5 to lo after infection /i.e. peak period of multiplication of E.stiedai by schizogony/, there was no practical value in giving medication either at the beginning of the parasite development /group 6/ or during gametogony. Although production of immature oocysts at the latter stage suggests some efficacy also at this time, gross lesions developed in the liver by about that time remained unchanged.

Analysis of the amount of drug consumed by rabbits in various groups suggests not only different susceptibility during the life cycle of E.stiedai but also implies that less drug is needed to control the parasite.

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ADVANCES IN HUMAN PARASITIC PROTOZOA

AMOEBIASIS, TOXOPLASMOSIS, MALARIA

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HOST-PARASITE INTERACTIONS IN AMOEBIASIS

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INTRODUCTION

The course of an infection with E. histolytica in humans is unpredictable depending on "host and parasite" factors. These factors are until now poorly understood and some of them will be discussed in this paper.

According to Sargeaunt and Green (1978), pathogenicity of E. histolytica is being defined by their invasive activity in humans. Such pathogenic strains of E. histolytica are characterized by the electrophoretic mobility of certain amoebic enzymes (Zymodemes). Apparently all the pathological changes are attributable to the powerful cytotoxic activity of the parasite. Several substances have been accused as causative agents such as an endogenous cytotoxin (Lushbaugh et al. 1979) and pore forming material (Lynch et al. 1982, Young et al. 1982, Ravdin et al. 1982). These cytopathogenic factors do not exert a systemic effect but depend on a close contact to their target cells: lectin-carbohydrate bindings seem to mediate this contact (Kobiler and Mirelman 1981, 1982). In this paper we support evidence that for effective killing of tissue culture cells only a few minutes' contact of living amoebae with target cells is necessary, thus confirming the "one hit" hypothesis of Ravdin (1980). Among pathogenic amoebae as defined by Sargeaunt and Green (1978), strains with different virulence can be distinguished. This can be done by means of the hamster liver infectivity test as well as by in vitro cytotoxicity assays, as will be shown in this paper.

Complement has been shown to exert an effective lytic action on trophozoites of laboratory strains of E. histolytica. The activation occurs mainly via the alternative pathway (Huldt et al. 1978, Stemberger 1978, Ortiz-Ortiz et al. 1978). The protective significance of these findings has been underlined by animal experiments (Ghardirian and Meerovitch 1982) and by the fact that the majority of strains of E. histolytica freshly isolated from liver abscesses were complement resistant (Reed et al. 1983). It has been shown in a previous paper that fresh normal human serum as a source of complement could completely suppress the cytotoxic action of E. histolytica in vitro (Hudler et al. 1983). In this paper we present evidence that complement and other serum factors cooperate in this respect.

MATERIALS AND METHODS

Amoebae

Trophozoites of E. histolytica of the strain HK9-AX were grown axenically in TYI-S-33 medium (Diamond et al. 1978), trophozoites of the strain SFL3-AC, HM1-AC and HK9-AC were grown in the same medium but monoxenically by the addition of crithidiae. Hamster liver infectivity tests as well as cytotoxicity assays were performed with culture harvested after 72 hours.

Complement

Fresh normal human serum which was negative in amoebae specific tests (IHA and IIFT) was used as a source of complement. The complement activity was 23 CH 50/ml.

Cytotoxicity assays were performed essentially as described by Hudler et al. (1983). Briefly, 0.2 ml of the target cell suspension (K 562, labelled with ^{51}Cr, $5x10^4$/ml) and 0.2 ml of medium or fresh normal human serum as a source of complement were mixed. After 10 minutes 0.2 ml of the suspension of amoebae (2.5×10^5/ml) was added. Trophozoites and target cells were pelleted by centrifugation (200 g for 2 min) and incubated at 37°C. After different time periods target cell lysis was

calculated from the radioactivity released into the supernatant. As a control radiolabelled K 562 was incubated with 0.2 ml medium instead of the trophozoite suspension (spontaneous lysis).

The "Virulence Index" was calculated according to the formula:

$$V.I. = \frac{\% \text{ lysis after } 30 \text{ min} - \% \text{ spontaneous lysis after } 30 \text{ min}}{\% \text{ lysis after } 180 \text{ min} - \% \text{ spontaneous lysis after } 180 \text{ min}}$$

Hamster liver infectivity test

Young male hamsters weighing 50-70 g were laparotomized and inoculated intrahepatically with 2×10^5 trophozoites suspended in 0.1 ml of sterile PBS. After one week liver abscesses were recorded.

RESULTS

In Fig. 1 the time course of the cytotoxic action of 3 laboratory strains of E. histolytica grown under different conditions (HK9 AX, SFL3 AC and A3 AC) against the tissue culture cell line K 562 is shown. The effector target cell ratio was 5:1. The most distinguishing feature between the two strains was the rapidity of onset of the cytolytic events: within the first 30 min, the axenically cultured HK9 was only able to effect a chromium release of about 15%, whereas within the same time period the monoxenically grown strains destroyed 60% of the target cells as could be calculated from the chromium release. After 90 minutes coincubation of amoebae and target cells, however, target cell lysis was in the range of 80% irrespective of the strain of E. histolytica used in the experiment.

From the data of these kinetic studies we calculated a "virulence index" (V.I.) according to the formula given in "Materials and Methods". In Table 1 the results of the hamster liver infectivity test of several laboratory strains of E. histolytica are compared with the corresponding V.I.'s. A significant correlation ($p<0.001$) was found between the in vitro and in vivo test.

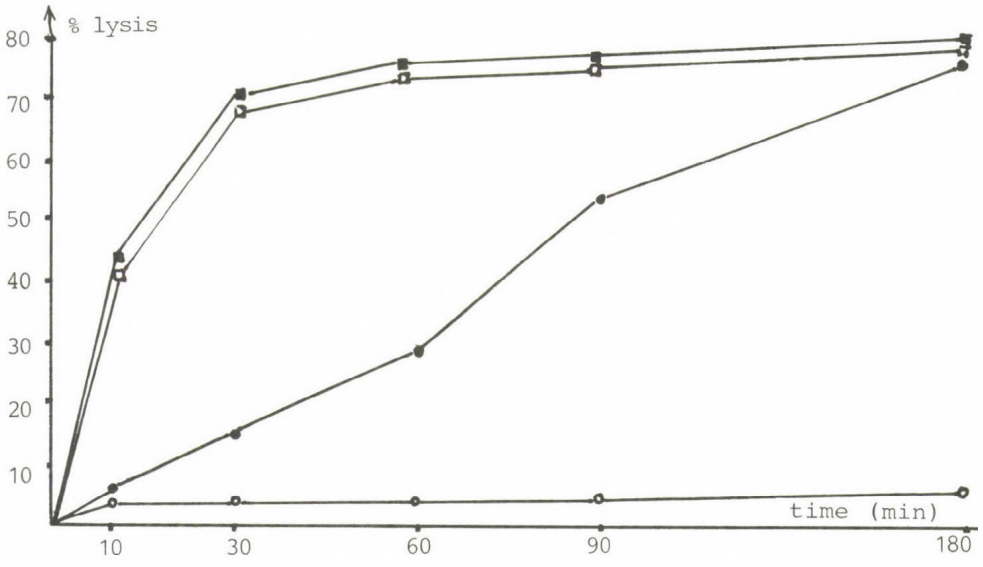

Fig. 1. Kinetics of target cell lysis by different strains of E. histolytica. ■—■ = SFL3 AC; □—□ = A3 AC; ●—● = HK9 AX; o—o = spontaneous lysis.

Table 1. Virulence index (V.I.) of several laboratory strains of E. histolytica versus results of the hamster liver infectivity tests

Strain	Culture condition	V.I.	Liver abscesses/hamsters infected
HM1	AC	0.87	8/8
SFL 3	AC	0.73	6/10
HK 9	AC	0.63	4/10
HK 9	AX	0.05	0/10

To elucidate the mode of the cytotoxic action of E. histolytica on tissue culture cells it was necessary to abolish cytotoxicity of amoebae selectively without damage of target cells. It is shown in Fig. 2 that this could be accomplished by fresh normal human serum (NHS) as a source of complement. The addition of NHS to amoebae at a final concentration of 6 CH 50/ml 10 minutes before addition of target cells reduced their

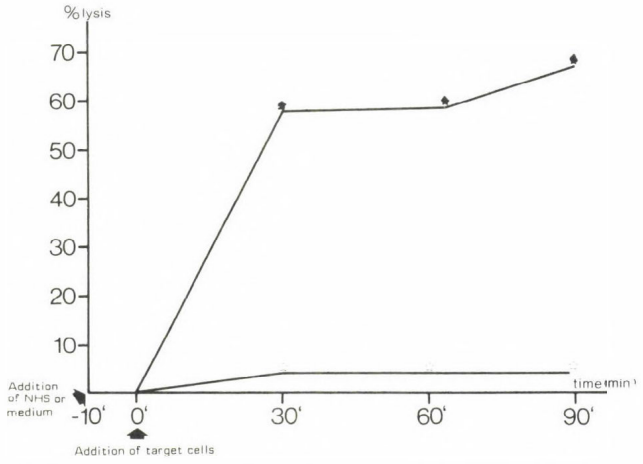

Fig. 2. Inhibitory effect of NHS as a source of complement on the cytotoxic effect of amoebae (SFL3 AC) against target cells. Amoebae preincubated with medium (●—●); amoebae preincubated with NHS (◇—◇).

cytotoxic activity to background levels during an observation period of 90 min.

In subsequent experiments 6 CH 50 of human complement were added after 10 minutes coincubation of a virulent strain of E. histolytica (SFL3 AC) and radiolabelled target cells. It is evident (Fig. 3) that in this assay complement was no longer able to prevent target cell lysis; the kinetic of target cell lysis was comparable to a control experiment in which medium was added instead of complement.

In the next set of experiments the influence of temperature on the time course of cytoxicity of E. histolytica was investigated.

For this purpose during the first 10 minutes of the experiment amoebae and target cells were incubated at +4°C. Thereafter they were incubated at +37°C or kept at +4°C, respectively and target cell lysis was checked at certain intervals during the following 80 minutes. It is shown in Fig. 4 that target cell lysis proceeded to high levels at a temperature of +37°C, whereas only insignificant lysis of target cells could be observed in the control assays kept at +4°C. Again, human

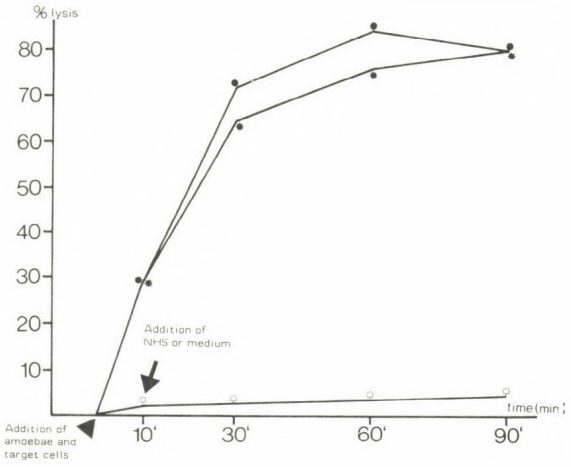

Fig. 3. Influence of NHS as a source of complement on the cytotoxic action of A3 AC against target cells. Complement (*—*) or medium as a control (●—●) was added after 10 minutes' coincubation of amoebae and target cells. ✡—✡ = spontaneous lysis.

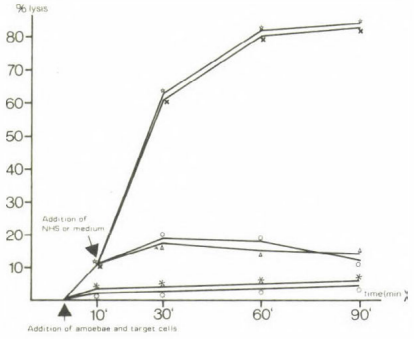

Fig. 4. Effects of temperature and NHS on the cytotoxicity of E. histolytica (SFL3 AC) against target cells. NHS was added after 10 min coincubation of amoebae and target cells at +4°C. Thereafter test tubes were incubated at +37°C (✡-✡with medium; x—x with NHS) or kept at +4°C (Δ—Δ with medium, o—owith NHS). ✪—✪ Spontaneous lysis at +4°C; *—* at 37°C.

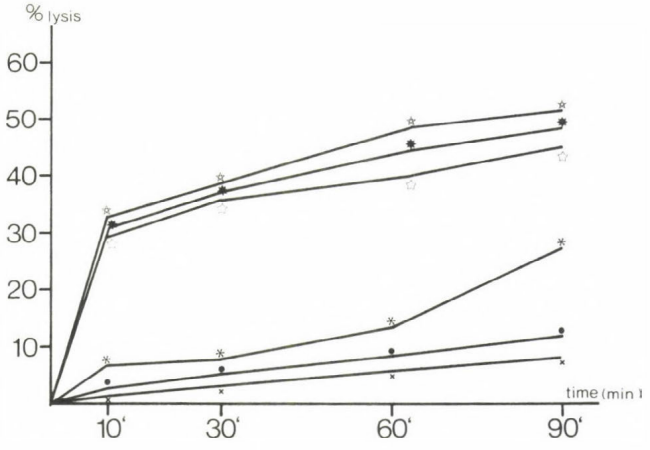

Fig. 5. Influence of NHS (●—●), heat-inactivated NHS (◇—◇), NHS treated with 10 mM EDTA (*—*), medium (✲—✲) and medium + 10 mM EDTA (✱—✱) on the cytotoxicity of E. histolytica (A3-AC) against target cells. x—x = spontaneous lysis.

complement could not influence the subsequent target cell lysis when added 10 min after the target and effector cells were brought into close contact.

The following experiment was designed to get insight into the nature of the inhibitory effect of NHS on the cytotoxic activity of E. histolytica. For this purpose amoebae strain A3 AC was admixed to either fresh NHS, heat-inactivated NHS, EDTA-treated NHS and medium as well as medium + EDTA as controls. 10 minutes thereafter target cells were added and lysis was checked at certain intervals for the subsequent 90 minutes (Fig. 5). Again, fresh NHS caused an almost complete suppression of amoebae-induced target cell lysis. On the other hand, EDTA in a concentration sufficient to abolish complement activation (10 mM) could only partly inhibit the suppressive effect of NHS on the cytotoxic activity of E. histolytica on tissue culture cells.

DISCUSSION

It has been shown that isolates of E. histolytica could be characterized according to the electrophoretic mobility of certain reference enzymes (zymodemes). There was an excellent correlation between the clinical symptoms caused by the isolated organism and its zymodeme type (Sargeaunt and Green 1978). It is now generally accepted, therefore, that amoebae can be classified into pathogenic and non-pathogenic strains according to their zymodemes. Amoebic strains maintained in long-term cultures and used for our experiments were all primarily isolated from clinical cases of invasive amoebiasis, including strain HK9-AX and are therefore to be classified as pathogenic strains. The results of the hamster liver infectivity test, however, revealed striking differences between these strains apparently due to the culture conditions under which they were maintained. We thus conclude that, apart from their pathogenicity, strains of different virulence can be distinguished by this in vivo test. In vitro, virulent strains were highly cytotoxic for tissue culture target cells within the first 30 min of coincubation, whereas the avirulent strain HK9-AX did not cause significant cell damage within this time. After 180 minutes' coincubation the virulent and avirulent strain finally reached a comparable degree of target cell damage. This observation indicates that both strains are basically pathogenic but of different virulence.

It has been shown by several authors that amoebae are being killed by normal human serum via the activation of the alternative pathway of complement (Huldt et al. 1978, Stemberger 1978, Ortiz-Ortiz et al. 1978). The amoebicidic effect of complement did never exceed 40-70%. The cytotoxic effect of amoebae on tissue culture cells, however, could be completely abolished by preincubation with normal human serum. Thus, the suppressive effect of complement on the cytotoxic activity of amoebae is by far more pronounced than could be expected from its direct amoebicidic effect. An explanation for this observation could be that the surviving amoebae are being blocked with respect to their lytic capacity, possibly not due to lethal alterations in the sense of surface redistribution of membrane structures

(Trissl et al. 1977, Aust-Kettis and Sundquist 1978, Calderon and Tovar-Gallegos 1980). In favour of this hypothesis speaks our observation (unpublished results) that amoebae, surviving complement treatment remained transiently resistant against newly added complement. As it could be shown in Fig. 5, complete inhibition of the classical and alternative pathway of complement by EDTA, the suppressive effect of NHS on cytotoxicity of amoebae, was only partly eliminated. It should be considered therefore that, besides complement, other serum factors may contribute to the NHS induced blockade of amoebic action. These factors apparently are partly heat labile (Fig. 4), since heat-inactivated NHS lost its suppressive effect to a considerable degree. These factors might be glycoproteins which interfere with the binding of effector to target cells and may therefore represent an additional humoral host defense mechanism against invasive amoebiasis. It has been shown by Kobiler and Mirelman (1980, 1981), and Ravdin et al. (1981) that such lectin-like interactions initiate the lytic events in the interplay between trophozoites and target cells.

It is well established that for effective lysis of target cells by E. histolytica a close contact between them is necessary (Ravdin et al. 1980). This author described a two-phase action of the pathogen:

1. a cytolethal phase
2. the phagocytosis of lysed cellular material.

By means of our chromium release assay the sequence of events during this first phase was studied (Figs 4 and 5). Already 10 min after coincubation of amoebae with target cells the sequence of cytolytic events could not be stopped anymore by the addition of NHS as a source of complement. As already mentioned, preincubation of amoebae with NHS completely abolished their cytotoxic activity. We conclude from these observations that the cytolethal phase can be subdivided into two stages: (1) contact dependent setting of the lethal hit for which the presence of active amoebae is necessary; (2) liberation of cytoplasmic material from the target cell (lytic phase) as measured by chromium release, which does not require the presence of active amoebae. The differentiation into two steps

is underlined by the fact that the first step proceeds at 4°C as well as at 37°C whereas the second step (lytic event) only takes place at 37°C.

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CULTURE MEDIUM FOR ENTAMOEBA HISTOLYTICA AND GIARDIA INTESTINALIS CONTAINING NUTRIENTS PRODUCED IN CZECHOSLOVAKIA

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The main obstacle in the efforts of scientists in socialist countries to engage themselves systematically in the diagnostic and theoretical problems of Entamoeba histolytica and Giardia intestinalis research is the dependence on the use of culture media containing ingredients imported from the USA or other western countries. To maintain axenic cultures of both protozoans, TYI-S-33 medium gives the best results (Diamond et al. 1978, Keister 1983). The defined portion of this medium consists of inorganic salts, amino acids and vitamins. The undefined portion is composed of 10 per cent of inactivated bovine serum and several nutrient biological products, i.e. enzymatic digests or extracts of casein, liver and yeasts.

In our experiments these undefined ingredients of TYI-S-33 medium have been replaced stepwise by the products of the Czechoslovak monopolized producer of dehydrated culture media - Imuna, n.p., Šarišské Michalany. The following four nutrients of this producer were tested: Enzymatic Casein Hydrolyzate, Acid Casein Hydrolyzate, Liver Hydrolyzate, and Yeast Autolyzate.

E. histolytica monoxenic culture with Crithidia sp. was derived in our laboratory from a polyxenic isolate obtained from the Marcinovski Institute in Moscow. To date this strain, designated as A, grows in more than 80 subcultures.

G. intestinalis axenic culture of the strain ATCC+30888 has been maintained in our laboratory in the TYI-S-33 medium for about two years. This culture was obtained from the London School of Hygiene and Tropical Medicine.

During the substitution experiments we had to keep under control both the total osmolality and pH values. The osmolality value, especially, had to be regulated with the decreased amount of sodium chloride in the medium because the Imuna products contain higher concentrations of inorganic salts.

As the result of our efforts, we can present the following modified formula of TYI-S-33 medium with two Imuna ingredients:

Enzymatic Casein Hydrolyzate Imuna	2	g
Yeast Autolyzate Imuna	1	g
Glucose	1	g
K_2HPO_4	0.1	g
KH_2PO_4	0.06	g
L-cystein HCl	0.1	g
L-ascorbic acid	0.02	g
ferric ammonium citrate	0.0022	g
distilled water ad	87.0	ml
pH adjusted to 6.8 with 1 N NaOH		
inactivated bovine serum	10.0	ml
bovine bile (for Giardia only)	0.1	ml
filter sterilized		

In the monoxenic E. histolytica cultures we did not observe any differences in the growth rates and total yields of amoebae in the modified medium when compared to the original one. Inocula of about 1 000 amoebae per milliliter of the medium are sufficient and about 10 to 20 times higher concentrations can be harvested. Cultures in screw-cap tubes can be transferred every third or fourth day. For the mass production of amoebic antigen we use the flat plastic flasks of 40 ml capacity. The purity of the antigen obtained is very good because the Crithidia are almost completely eliminated during the 72 hours of incubation.

G. intestinalis grows abundantly in the modified medium with Imuna ingredients. The subcultures require slightly higher inocula than cultures in the original medium. Transfers can be performed at 7 day intervals since the generation time of Giardia is prolonged to approximately 12 hours. However, suspensions of highest purity and concentration can be obtained from this medium either for immunological or biochemical studies.

In discussing our results we would like to emphasize that many problems connected with the successful cultivation of G. intestinalis, and especially E. histolytica, are far from being solved. However, tested modified media from our commercially produced ingredients do ensure the possibility of axenic and monoxenic maintenance of these protozoans independentely of the imported products. This we consider an important precondition for the further development of research activities in related fields.

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STUDIES ON THE DIAGNOSIS AND TREATMENT OF AMOEBIC UVEITIS

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Amoebic dysentery is not usually associated with uveal symptoms, but the possibility of its occurrence has been shown by the animal experiments of Vozza (12) who found that Entamoeba histolytica inoculated into the anterior chamber of a rabbit's eye can cause a severe plastic and haemorrhagic iridocyclitis.

Endogenous uveitis observed in recent years was not only associated with toxoplasmosis or toxocariasis but with Entamoeba histolytica infections as well.

In review of the literature, workers from all over the world have reported cases of uveitis with intestinal amoebiasis. The first case of septic iridocyclitis associated with amoebic dysentery was studied by Houdart in 1904 (3); since then many cases have been recorded. Anterior uveitis with intestinal amoebiasis was reported by Samaviejo (10), Paul (9) and Sarda (11). Others, like Krummel (7), Braley (1), Harris (2), King(6) gave accounts of posterior uveitis.

We could not find any data referring to ocular manifestation of amoebiasis in the Hungarian literature.

The aim of this work was to study the occurrence of amoebiasis associated with uveitis in our country and to draw attention of ophthalmologists and parasitologists to the clinical characteristics, diagnostic criteria and therapeutic responses of the disease.

We studied 293 cases of endogenous uveitis from an Ophthalmologic Clinic. The suspected cases were investigated by different methods.

As a first step other causes of endogenous uveitis like toxoplasmosis or tuberculosis with either routine or special investigations were ruled out. The 3 consecutive samples of stool were examined by the Riedley and Faust concentration technique for E. histolytica. The stool samples were cultured with Dobbel-Laidlaw diphasic media and each uveitic patient had an indirect HA test for amoebiasis (by Cellognost, Behring test). Our results are summarized in Fig. 1.

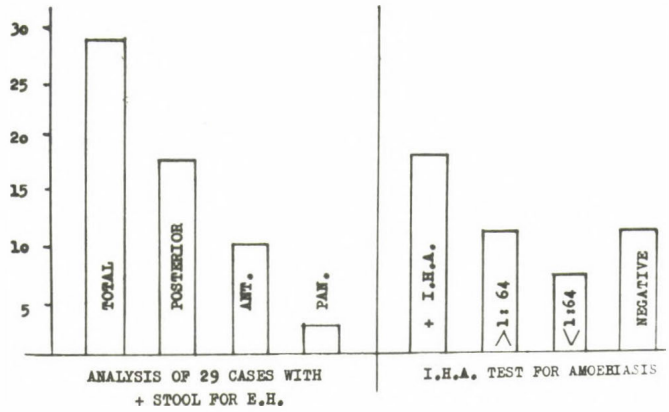

Fig. 1. Clinical pattern of amoebic uveitis.

Twenty-nine cases had a positive stool for E. histolytica, anterior uveitis was seen in 10 cases, posterior uveitis in 17 and panuveitis in 2. Eighteen patients had a positive indirect HA. In 11 cases the titre was higher than 1:64.

Table 1. Clinical appearances of amoebic uveitis

Anterior uveitis			Posterior uveitis	
	Haemorrhagic	Non-haemorrhagic		
Non-granulomatous	3	1	Focal	12
			Diffuse	4
Granulomatous	-	6	Disseminated	1

With few exceptions the symptoms we found were very similar to those which were described by others (8). However, we observed cystic peripheral haemorrhagic chorioretinitis causing massive exudate in the vitreum as well as pars planitis. This occurrence usually is thought to be caused by Toxocara infection. In another case recurrent vitreous haemorrhage developed following chorioretinitis.

Apart from the 29 cases we have presented in Fig. 1, we found a few other cases where the stool examination result was negative, but the IHA test was positive and the clinical occurrence was very similar to the above-mentioned cases; we could still achieve good clinical results by using antiamoebic therapy.

For therapy we used the well-known antiamoebic drugs metronidazole, tinidazole combined with wide spectrum antibiotics. The currently available preparations of metronidazole do not always meet the different therapeutic requirements. The use of tablets is often impractical.

We prepared a suppository which could be used effectively in cases of oral treatment intolerance and also in other clinical situations. This suppository was used earlier with a 94% therapeutic success rate in giardiasis (5).

After we found the very suppository base - Massa Estarini 299 - which would give us the most effective release of the active substance, we applied this preparation in healthy volunteers.

The suppositories have given sufficiently high serum levels of metronidazole, and the serum concentration was in the range of antiamoebic effectivity (4).

The serum concentrations of rectally administered metronidazole (Fig. 2) reached the peak more slowly than in the cases of oral dosing; t max. was between 4 and 6 hours. The average peak concentration is 9.5 mg/l.

Considering the bioavailability of metronidazole, we used suppositories containing 500 mg metronidazole 4 times daily for 7 days to the patients with uveitis.

Of the patients 72.4% showed a good therapeutic response. After a course of therapy with metronidazole the stool tests for

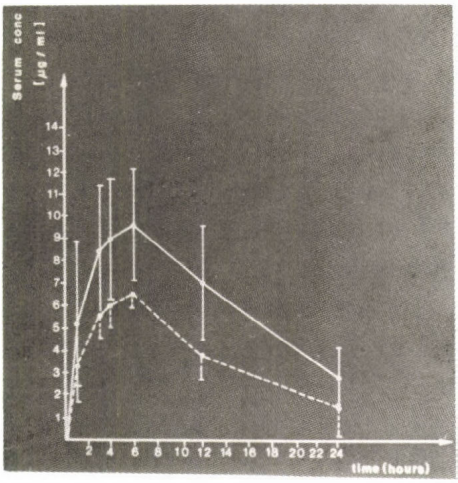

Fig. 2. Serum levels of metronidazole after 500 mg rectal suppository.

Table 2. Therapeutic response to metronidazole

Type	No. of cases	Cured	Recurrences	No response
Anterior uveitis	10	7	2	1
Posterior uveitis	17	13	1	3
Panuveitis	2	1	-	1
Total	29	21 (72.4%)	3 (10.3%)	5 (17.2%)

E. histolytica became negative. 17.2% of the patients showed no response to this drug. In 3 cases we observed recurrences.

Summing up our results we can state that 9.9% of our 293 patients had uveitis associated with intestinal amoebiasis. In the early stages anterior uveitis showed an acute non-granulomatous lesion with a haemorrhagic exudate. Subacute and chronic cases showed the intermediate type and later the typical granulomatous type of lesion. Haemorrhagic exudates in the anterior chamber and haemorrhages in and around the chorioretinal lesions are also a characteristic finding in amoebic uveitis.

The diagnosis was established on the basis of characteristic clinical appearances and E. histolytica in the stool specimen and/or positive indirect HA for E. histolytica; and a good therapeutic response.

We examined and investigated the ocular manifestation of amoebiasis for the first time in Hungary, and in 3/4 of the cases the patients responded well to the metronidazole suppository therapy.

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OCCURRENCE OF ENTAMOEBA HISTOLYTICA IN COUNTY BORSOD (HUNGARY) FROM 1980 TO 1984

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Entamoeba histolytica is a common parasite in the intestine of man. It is the causative agent of amoebic dysentery. Intestinal and extraintestinal forms are known (1).

The data collected by the National Institute of Hygiene (OKI) during the past 3 years shows that of 130,000 to 140,000 people examined in Hungary, 0.6% were infected in 1980, 0.49% in 1981 and 0.5% in 1982 (2). In the county of Borsod, we have been conducting regular protozoological tests for about 30 years (Table 1).

TEST METHODS

All material sent to our laboratory for protozoological examination is prepared as a smear in physiological saline and examined microscopically. Stools suspected of harbouring E. histolytica are inoculated into Bork-Debochlav culture medium (3). If this test gives negative results but clinical conditions still suggest amoebic dysentery, a series of tests will be carried out. We have found that even if the third test is negative, the clinical symptoms persist. In the case of screening subjects at their homes, only a single examination is made. The validity of our results is also questioned because many samples arrive by mail and may only be tested several days after sampling.

E. histolytica appears not to be very prevalent in the county of Borsod. This may be because it is subject to a tem-

Table 1. Occurrence of infection between 1980-1984

	1980	1981	1982	1983	1984
Positive, total	73	48	40	73	100
Cyst	45	35	19	22	31
Grown	28	13	21	51	69
Number of persons tested	15,353	15,596	14,264	15,802	13,629
Positive %	0.47	0.31	0.28	0.46	0.73
Clinically registered %	17.80	20.80	22.50	20.30	19.00

The territorial distribution of infection is shown in Table 2.

Table 2. The geographical distribution of infection

	1980	1981	1982	1983	1984
Miskolc	30	16	11	27	42
Other towns	9	7	4	9	15
Villages	34	25	25	37	43

perate, and not a tropical climate. The number of recorded infections is greater from towns than from rural areas. This is because more people are examined is towns. In Miskolc (Northern Hungary) there are close links between the clinical department and this laboratory. The greatest incidence of infections is with children aged from 0 to 10 years. After 10, the number of infections is reduced.

The results of laboratory culture tests can be of great help to the physician in diagnosing amoebiasis. Information may be provided on the size, shape and pattern of movement of amoebae in culture. Clinicians may then establish if this information matches their diagnosis. Initially, 400 to 500 culture tests were made each year but as the tests are very time-consuming, this number has been reduced to 200.

Positive results from smear tests were usually obtained with successful cultures, but there have been some cases when the cultures remained negative despite the presence of cysts in stools. Table 3 shows the incidence of successful cultures.

Table 3. Results of cultures

	1980	1981	1982	1983	1984
Number of cultures	188	121	117	209	248
Positive, %	15.9	10.7	20.5	26.3	30.6

Many metacystic forms may be found in one third of the cultures. Sometimes at 24 hours, the time of the first examination, Entamoeba coli was also present. Only in 20 to 25% of the second subcultures could metacystic forms be found. Eventually, E. coli will prevent the growth of E. histolytica in culture. Moving cells can then be found only rarely.

The optimal pH for growth in culture is 7.0 to 7.4. Strains may usually be maintained for 3 to 4 weeks in culture, but some may survive for 2 to 3 months. This may happen once or twice a year. Cultures taken from children's samples typically survive better.

The protozoa are fed with washed human blood cells. This makes the amoeba more active but also accelerates their degeneration. As a result of cell degeneration, the pH of the medium becomes more acidic (pH 5.8-6.2). By this time the culture becomes thick and smells badly.

During our work we have encountered both acute and chronic cases. Acute cases occurred mostly at the 2nd Department of Infectious Diseases, Semmelweis Hospital, Miskolc. Patients were admitted with enteric disorders. Amoebiasis sometimes occurred in conjunction with bacterial dysentery.

Chronic cases are less common. Such cases are treated at the Dermatological Department. Symptoms include long-lasting and often recurrent colitis, ulcers, hepatitis, hepatosis and chronic dermatitis with sores. Abdominal complaints and allergic dermatological disorders were also characteristic symptoms in children. Most cases were without symptoms.

Several cases of subjects who were passing cysts but who had no symptoms were encountered among children at the Children's Home at Tokaj and among workers from the waste purifying works.

The incidence of E. histolytica in stools of subjects returning from tropical countries is as follows: 1982 - 1 case, 1983 - 4 cases and 1984 - 100 cases. The numbers of relapses noted since 1980 were: 1980, four; 1981, one; 1982, two; 1983, one and 1984, one. During this time, a number of drugs were used for the treatment of amoebiasis. They included Yatren, Resochin, Resotren, Milibis, Enteroseptol, Delagil, Neoviasept, Emetin, Dehydroemetin and Fumagilin. Currently, we use Metronidazole, Ornidazole or Tinidazole, three times daily in 1200 to 1400 mg doses for 10 days. 0.73% of the patients recover. If recovery is slow, 2 x 250 mg Chloroquine may be given daily.

Control examinations, made 4 to 8 weeks after recovery, sometimes present problems, especially with those patients who did not exhibit any symptoms. We try to perform control examination by involving clinical workers and the epidemiological network.

These results relate to the last five years in Hungary. We have found a lot of variations associated with E. histolytica. Symptoms are very varied as to the responses of the organism to culturing. We find that the disease occurs sporadically in small endemic centres such as families or children's communities. It would be useful to have a system of serological tests to extend our knowledge of this organism in Hungary. This is not possible at present because antigens are not available. With such a scheme we could aid clinical workers in eradicating amoebiasis.

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LABORATORY CONTRIBUTIONS TO THE DIAGNOSIS OF TOXOPLASMOSIS

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We cannot entirely rely either on the anamnestic data or on the clinical symptoms in establishing the diagnosis of toxoplasmosis. The scale of infections caused by the cysts and oocysts of Toxoplasma gondii is quite wide. Sources of infection may be contacts with cats, environmental pollution and consumption of raw or not satisfactorily heat-treated meat, too. The clinical symptoms of toxoplasmosis are as follows: slightly sore throat, nasal discharge, lymphadenopathy, ocular lesions etc. They may appear in the form of viral and bacterial infection, too. To verify the disease in question, the clinicians are to rely on laboratory examinations. Among them demonstration of the parasite, inoculation of various kinds of material into mice, histopathologic as well as immunodiagnostic examinations are of greatest help. The most important of these methods are the serologic reactions: the Sabin-Feldman test, the complement fixation reaction /CFR/, the indirect haemagglutination /IHA/, the indirect fluorescent antibody test /IFA/ and the ELISA . Depending on the sensibility of the method, higher or lower concentrations of the antibody found in the serum can be shown.

In verification of toxoplasmosis we mainly used serodiagdostic examinations. The antigen was made of the peritoneal exudate of mice infected with RH strain of T.gondii. The dose was determined in relation to the value of some other commercial antigens. We carried out comperative examinations

observing the progress or regression of the disease with the help of different seroreactions. The results showed that the slope of the immunogram of the CFR and the IFA was steep and that of ELISA and IHA was flat. In the light of the above, we chose the CFR and the IHA for our examinations to differentiate the acute from the latent disease by determining the IgM treating the sera with mercaptoethanol or by seroconversion.

In our lecture we give account of the examination of 5358 patients suspected of toxoplasmosis. On the basis of the clinical symptoms, the patients were divided into four groups: lymphadenopathic toxoplasmosis, ocular toxoplasmosis, intrauterine T.gondii infection and other kinds of toxoplasmosis. The distribution of the pathologic picture is shown in Fig. 1.

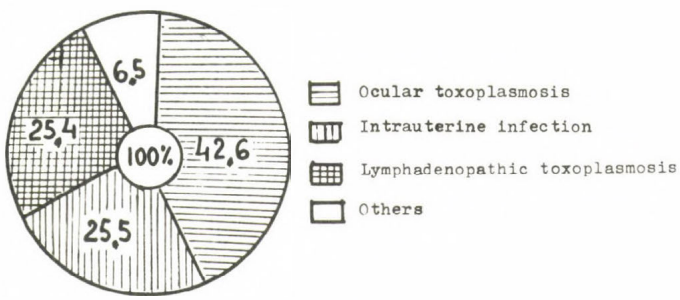

Fig. 1. Distribution of diagnosis in suspected cases of toxoplasmosis

42.6% of the serologically examined patients had a supposed diagnosis of ocular toxoplasmosis. Then follow in the order of frequency intrauterine infections 25.5%, lymphadenopathic toxoplasmosis 25.4% and other kinds of toxoplasmosis 6.5%.

The positivity of the CFR, i.e. the frequency of the acute and subacute cases is demonstrated in Fig.2.

The complement fixing antibody was registered the most frequently in 29.5% of all cases among the patients suspected of lymphadenopathic toxoplasmosis, the least frequently it appeared in the other toxoplasmosis groups.

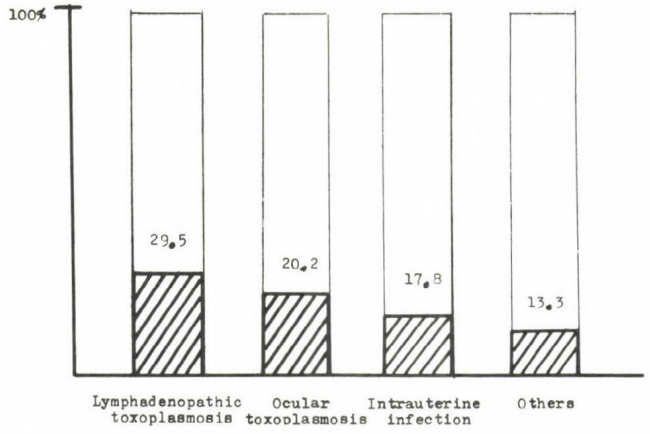

Fig. 2. Incidence of seropositivity according to complement fixation test

In Table 1, we summarized the division of the seropositivity according to high - above 1:10 - and low - below 1:10 - values of complement fixing antibodies.

The high titres - probably the new infections - appeared the most frequently in 61.4% of those with lymphadenopathic toxoplasmosis. In ocular toxoplasmosis and intrauterine infections the lower values 80.1% and 85.2% predominated.

In lymphadenopathic toxoplasmosis high values of titre were registered at the age group of 11-30 years. As a result of our seroepidemiological examinations, we found that the frequency of Toxoplasma infections was also the highest at this age group. It is clear that the adenopathy often following the T.gondii infection is also registered at this age. The contamination of women is 10% higher than that of men. This fact may be explained by two reasons:

1. Having noticed the collum adenopathy characteristic of this disease women consult sooner the doctor.
2. In the course of different kinds of housework women are more exposed to the infection.

With ocular toxoplasmosis high values of complement fixing antibodies are usually detected in the blood sample of children while lower values are found in the older genera-

Table 1. Distribution of seropositivity according to antibody titres

Clinical forms	Antibody titres				Total
	< 1:10		> 1:10		
	No.	%	No.	%	
Lymphadenopathic toxoplasmosis	172	38,6	273	61,4	445
Ocular toxoplasmosis	321	80,1	80	19,9	401
Intrauterine infection	258	85,2	45	14,8	303
Others	15	/78,9/	4	/21,1/	19
Total	766	66,4	402	34,4	1168

tions. In 90.0% of the patients examined on the basis of ophthalmic diagnosis haemagglutinating antibodies were found, the positivity was higher than 59.6% shown in the healthy population. Acute toxoplasmosis was determined in 4.9% of the patients. In most cases the disease probably resulted from intrauterine infections.

In the group of the patients suspected of intrauterine infection complement fixing antibodies were found in 158 newborn babies. In 2/3 of the cases the antibodies were produced not by the fetuses themselves but they resulted from the infection of the mother. The diagnosis of the connatal toxoplasmosis could be proved merely in 5 newborn babies by the presence of IgM and the rise in titre conforming to the clinical symptoms.

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CLINICAL PROCESS AND TREATMENT OF OCULAR TOXOPLASMOSIS

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SUMMARY

The authors present a case of ocular toxoplasmosis in adult age and describe the clinical process, diagnosis and treatment of the disease. They verify that the treatment of the disease in adults can be performed with complex therapy - antitoxoplasma drug treatment, cryopexia, argon-laser photocoagulation. The patient has been well for a year now and can do her work.

Toxoplasmosis, a widespread infection in both man and animals is caused by the intracellular protozoan parasite, Toxoplasma gondii (11,15). The disease can be acquired congenitally or by ingestion (1,2,10,12).

Serological data indicate that about 30% of the United States population has been infected with this organism (cit. 4). Higher incidences have been found elsewhere. In Hungary the rate of incidence is 52.9% among population under the age of 60 (9).

About one-third of the clinically evident Toxoplasma gondii infections in pregnant women result in congenital transmission of the disease (cit. 4). In the case of Hungary this means that about 300 congenitally infected babies are born every year (16). During the period from 1981 to 1984 we examined 32 patients with toxoplasmosis. We found two patients with ocular lesions. One of them was a Syrian woman, who returned to her country after the first drug treatment, so follow-up became impossible. Another one, A.S., was a Hungarian woman.

REPORT OF THE CASE

A.S., a 23-year-old female patient, has been in our care since the age of 20. Her sight deteriorated in her left eye during pregnancy at the beginning of 1982. Juxtapapillary choroiditis caused the decrease of visual acuity to 5/15. In the fundus fluorescein angiogram it is clearly shown that the papillary border is not sharp, especially nasally it became blurred: the vessel walls leaked the dye, thus causing a large cloud-like fluorescein extravasate before the optic disc during the late venous phase (Figs 1 and 2).

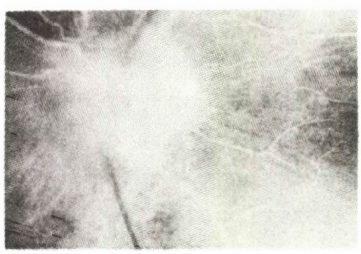

Fig. 1. Fundus fluorescein angiogram in arterial phase

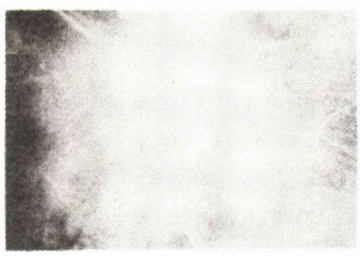

Fig. 2. Fundus fluorescein angiogram in late venous phase

The patient is clinically well, there are no signs of systemic disease like lymphadenopathy, fever or organic lesions. We did not find symptoms of syphilis or tuberculosis.

Blood count, thrombocyte and sedimentation tests show normal values, there are no signs of bone-marrow deficiency. Serum electrophoresis and immunoelectrophoresis are negative, cytotoxicity activity is one-third of the normal (Table 1). Enzymes are normal, no signs of organic lesion can be found. Toxoplasma serological reactions are positive in small titre, but VDRL is negative. Mantoux reaction is normal (patient is vaccinated by BCG). In the eye local antitoxoplasma antibody production can be detected, aqueous humour antibody coefficient is positive (Table 2). At the onset of the disease only drug treatment was undertaken (from 1982 to September 1983) in the following way: Spiramycin 3000 mg/day, pyrimethamine 75-25 mg/day (decreasing

Table 1

Time	Complete blood count						Thrombocyte	Sed. rate*	Serum electrophoresis (g/l)				
	Hb (mmol/l)	WBC ($1000/mm^3$)	Pa (%)	Se (%)	Ly (%)	Mo (%)	($10^9/l$)	(mm/h)	a	α_1	α_2	β	γ
March 1982	8.6	7.8	2	56	36	6	160	8	40	2	8	6	10
Sept. 1983	8.9	6.2	0	66	30	4	220	5	42	3	6	6	12
Nov. 1983	8.8	6.4	1	60	34	5	200	4	40	1	5	5	14
March 1984	9.0	6.0	0	68	30	2	220	3	45	1	6	5	12
March 1985	9.2	6.6	0	70	28	2	240	2	46	2	6	8	10

*According to Westergren.

doses), sulphamethoxydiazine 500 mg/day - by supplementary administration of folinic acid - during the period of 18 months with 2-3 weeks' interruption.

Steroids were only given to control acute exacerbations: high initial doses (loading doses) were given intravenously for a short time (5 days), then the dosage was reduced.

By September 1983 the posterior segment inflammation healed after a fluctuating course of the disease. By this time there was no fluorescein diffusion in the fundus angiogram, however, scattered erosions in the pigment epithelium due to inflammation provide windows on choroideal and scleral fluorescence. One month later, however, the disease reappeared in a new ophthalmological form, i.e. as inflammation in the area of the pars plana. The visual acuity of the left eye redeteriorated to 5/12 because of inflammatory foci at 5 and 8 o'clock positions.

The large vitreous opacities caused by these foci are shown in the red reflex through the pupil.

Table 2

Time	Immuno-electrophoresis (mg/100 ml)			Enzymes (U/l)			Toxoplasma serologic reactions		
	IgG	IgA	IgM	SGPT	AIP	γGT	IFA (IU/ml) IgG type	ELISA (IU/ml)	Aqueous humour AB coeff.
March 1982	1300	240	80	18	102	22	1:1250	494	12
Sept. 1983	1200	240	85	16	90	20	1:250	208	6
Nov. 1983	1300	250	90	18	96	18	1:250	260	8
March 1984	1200	240	88	20	90	16	1:250	202	6
March 1985	1200	250	90	15	90	16	1:250	98	2

The anterior segment inflammation was treated by antitoxoplasma drug treatment in a short time following the acute attack and by intensive transcleral cryopexia.

Relapses occurred three times at about 6 months intervals. The individual recurrences were less serious than the first anterior segment inflammation and could be managed by repeated cryopexia. The minimal exudation could finally be isolated by direct argon-laser photocoagulation.

METHODS

We used immunoserological methods for diagnosis and continuous observation of the activity of the Toxoplasma gondii infection: indirect immunofluorescence test (with Bio-Merieux antigen) and ELISA (Cordia T 100-Test Set for Toxoplasma antibodies), an enzyme labelled immunoassay for the quantitative determination of antibodies.

We examined in both the serum and the aqueous humour the level of antibodies: we determined the aqueous humour antibody coefficient by Desmonts' method (6).

DISCUSSION

Now let us discuss the clinical process, diagnosis and treatment of eye toxoplasmosis in adults with reference to a severe case at our hospital. We found this case worth presenting as it is not a rare condition but one that affects a large percentage of the population all over the world. At least 30-40% of all cases of chorioretinitis from 5 to 40 years of age are caused by Toxoplasma gondii. The most frequent cause of posterior uveitis is toxoplasmosis (14).

Our patient suffered from a severe form of uveitis, which threatened the sight of her left eye. The anti-toxoplasma antibodies of the IgG type were present in a small titre in her serum, IgM type antibodies were not detected. The aqueous humour had an antitoxoplasma antibody greater than the serum; this means that the ocular disturbances had a toxoplasmic aetiology. Still, during the activity of eye-disease signs of systemic illness were not found. Most of the adolescents and adults found to have congenital toxoplasmosis had only ocular lesions (5). It is typical and corresponds to data in the literature that the activation of the disease may occur at any time of life and can be induced by a change in the immunosystem of the organism, in this case by pregnancy (4, 13).

The cytotoxic activity is one-third of the normal, this means the deficiency of the cell-mediated immune response of the patient. A specific feature of the case was that the process was juxtapapillary, changing in location during the treatment and recurring as pars planitis. We think that the activation of the disease later in life can only be prevented by early medication in the very first year of life (7, 8).

The treatment of the disease activated in adult age requires long-term administration of high-dose drugs with hazards. Pyrimethamine sulphamethoxydiazine combination is a folic acid

antagonist that produces depression of the bone-marrow (thrombopenia, leukopenia and megaloblastic anaemia (3,8). We did not find side-effects because of preventive administration of folinic acid.

As a result of 18-month intermittent therapy, the chorioretinitis was cured. Definitive results can be obtained with complementary therapy (cryopexia, argon-laser photocoagulation). The patient is well now, she has not had any relapse since September 1984, can perform her work and has no complaints.

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COMPARISON OF THE PELLICLES OF DIFFERENT STAGES OF MALARIA PARASITES USING FREEZE-FRACTURE TECHNIQUES

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INTRODUCTION

The use of freeze-fracturing techniques in studies of the ultrastructure of the malarial parasite has yielded some unique morphological information unobtainable at present with any other method, and has now been used to study virtually all the stages of the life cycle of the parasite. But there are still areas where more research is needed. The early stages of gametocyte and sporozoite formation have not been studied by this technique and there is only a brief report on the exoerythrocytic stages by Meszoely et al. (1975).

Most of the above studies have concentrated on the architecture of the pellicular system of the parasite. During the process of freeze-fracturing, the various membranes of the complex are split along their hydrophobic interior, each membrane yielding two fracture faces. The fracture faces are covered by intramembranous particles, which differ in density and pattern from one membrane to the next and which distinguish the various intramembranous faces. A brief summary of the observations on various stages of the parasite life cycle is given below.

OBSERVATIONS AND CONCLUSIONS

Sporozoites

(Fig. 1A) Both the immature (Dubremetz et al. 1979; Meszoely et al. 1982) and the mature sporozoite (Aikawa et al. 1979) have been studied with the aid of freeze-fracture techniques. The pellicular system of these various sporozoite stages is virtually identical, consisting of three membranes and a row of subpellicular microtubules. A unique arrangement of intramembranous particles occurs in this stage. On

two out of the six fracture faces the particles are arranged in distinct parallel rows (Fig. 2) along the long axis of the parasite. The significance of this arrangement is not clear, but relationship to the underlying microtubules has been suggested (Dubremetz et al. 1979). This may in turn relate to motility or the cytoskeleton of the sporozoite. A unique structure found exclusively in the sporozoite and observed only in fractured preparations is a single longitudinal suture line that cuts through the middle and inner membranes of the pellicular complex (Fig. 2). Dubremetz et al. (1979) suggested that this suture is formed during the early development of sporozoites and marks the point of fusion of the vesicle from which the middle and inner membranes are derived.

Asexual erythrocytic stages (Fig. 1B)

The free merozoite is surrounded by three membranes as well as a set of microtubules (McLaren et al. 1979). However, the architecture of the pellicular system differs markedly from that of sporozoites. In the free merozoite none of the fracture faces displays the orderly arrangement of intramembranous particles seen in sporozoites. On all the fracture faces these particles are randomly distributed although they differ from one another in particle density and size.

As the free merozoite invades the erythrocyte it carries with it the host erythrocyte membrane. Thus, the outer membrane of the intraerythrocytic parasite (=parasitophorous vacuole membrane) is actually of host origin. The parasite also loses two of its former three membranes, the middle and inner pellicular membranes. Thus, the trophozoite is surrounded by two membranes, an outer vacuolar membrane of host origin and a second membrane which is the outermost membrane of the free merozoite pellicle. The fracture faces of both of these membranes undergo particle redistribution and in the mature trophozoite the architecture of the fracture faces differs considerably from the membranes from which they were derived (McLaren et al. 1979).

Gametocytes (Fig. 1C)

The ultrastructure of the gametocyte has only recently been investigated by freeze-fracturing (Meszoely et al. 1983, 1985). Its pellicle also consists of three membranes and a set of microtubules with an intramembranous particle distribution similar to that of the free merozoite. This is surprising since the free merozoite outer

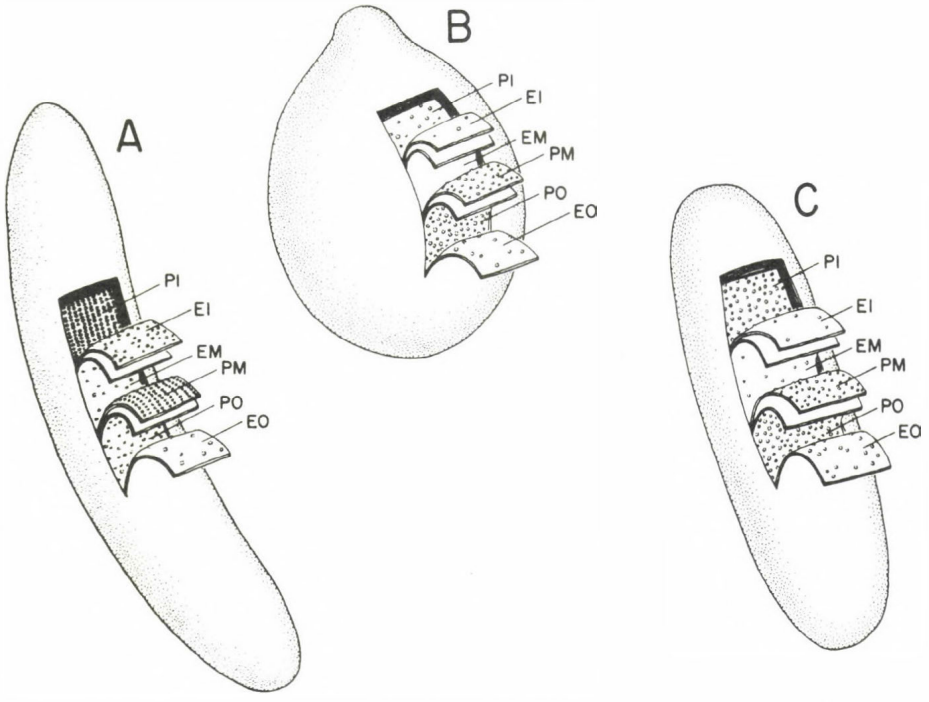

Fig. 1. Diagrammatic reconstruction of the pellicular system of three stages in the life cycle of the malaria parasite: (A) sporozoite; (B) free merozoite; (C) gametocyte; EO, external face of the outer membrane; PO, protoplasmic face of the outer membrane; PM, protoplasmic face of the middle membrane; EM, external face of the middle membrane; EI, external face of the inner membrane; PI, protoplasmic face of the inner membrane. Note that in (C) the PO and EO represent the fracture faces of the vacuolar membrane, derived from the host erythrocyte.

membrane is reported to become the gametocyte middle membrane as the parasite invades the erythrocyte and carries the host's plasma membrane with it, transforming it into the outer membrane of the pellicular system of the gametocyte. Little is known about the early immature stages of the gametocyte, but considerable rearrangement of intramembranous

Fig. 2. Plasmodium falciparum sporozoite showing three concave fracture faces: O, outer membrane; M, middle membrane; I, inner membrane. Note the scalloped suture line following the long axis of the sporozoite. Arrow points to a cytostome.

Fig. 3. Plasmodium falciparum gametocyte showing three convex fracture faces. For abbreviations, see Fig. 2. Arrows point to suture.

particles would have to take place in these membranes for them to be in fact homologous. A unique discovery in freeze-fractured gametocyte preparations was the presence of several transverse sutures on the middle membrane which appear to encircle the gametocyte giving it a segmented appearance (Fig. 3). The origin or function of these transverse sutures is not yet known. Clearly, the points just raised require clarification before the pellicular complex of the gametocyte and its homology to those of other stages can be resolved.

The evidence from freeze-fracture studies thus far strongly suggests that major rearrangements of the parasite pellicular membranes occur from one stage to the next and that the parasite pellicle exhibits a stage-specific architecture which is related to specific function and location in the host. This conclusion is supported by the observations that surface antigens of these different parasite stages also appear to be unique for each stage.

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THE SIGNIFICANCE OF MEASURING ANTIMALARIAL ANTIBODIES IN THE EFFICACY OF CHEMOPROPHYLAXIS

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Malaria is one of the most serious and most widespread tropical diseases. According to WHO statistics the disease had been suppressed in the sixties and in the early seventies, but since the middle of the seventies it is claiming more and more victims again all over the world.

The most effective way against malaria for people travelling to malaria-endemic areas in our days seems to be chemoprophylaxis. These days the 4-aminoquinoline derivates /chloroquine, amodiaquine, etc./, the antifolic pyrimethamine, and proguanil seem to be the most widely used chemoprophylactics (1,2).

The growing resistance of the Plasmodium, primarily that of P. falciparum (3), makes it necessary to search for new, more effective antimalarial drugs and to introduce them into chemoprophylaxis (4).

The latter - besides the serious side-effects of the known drugs /for example the combined antifol preparates/ - brings the danger of the appearance of resistance to the drug with it. These facts have also contributed to the publication of an increasing number of papers dealing with malaria chemoprophylaxis, nevertheless a common view has not been reached yet (5,6).

Since the middle of the sixties the number of Hungarian subjects travelling to malaria-endemic areas and working there for a long time has been increasing gradually and has had a sudden increase in recent years which highlights the importance of malaria surveillance. The compulsory chemoprophylaxis was introduced for persons travelling to malaria-endemic areas as well as the compulsory screening examinations on returning.

In this paper we present the retrospective data obtained from 7oo Hungarian subjects having lived in malaria-endemic areas for more than six months. Naturally the probability of infection was uneven since it varies in different countries. And above that it is natural that, for example a Hungarian diplomat serving in a malaria-endemic country is less likely to contact malaria than an agricultural specialist working in rural areas.

Method

We obtained our retrospective data from several sources. First of all from interviews. We asked the persons in questionnaires for detailed information about the time they spent abroad, the chemoprophylactics they used. We focussed on the incidence of malaria and especially on the results of parasitic examinations. Our main aim was to learn whether the patient having fever was treated and he or she recovered due to antimalarials. Physical examinations focussed on the presence of hepatosplenomegaly. Apart from the obligatory laboratory examinations, blood film was checked for parasites, and also serum was checked for the presence of specific antimalarial antibodies.

This latter was carried out by using P. falciparum IFAT Spot (7) test manufactured by Bio-Merieux according to the instructions. For coupling the specific antibodies fluorescein isothiocyanate labelled anti-human IgG and IgM goat serum was used by Human Pharmaceuticals. The tests in all cases were carried out simultaneously. For measuring of specific IgM one to ten

and one to twenty serum dilutions and of IgG one to twenty and one to eighty serum dilutions were used.
For monitoring Olympus HB-2 microscope with FITC adapter was used.

Results

Table I shows the data of 7oo persons taking different types of chemoprophylactics. From the chloroquine taking group 146 persons took regularly two tablets of Delagil obtained from Alkaloida Chemical Works, containing 15o mg chloroquine bases. In this group 2 parasitologically verified malaria cases occurred, whereas in 11 cases there were no parasitological examinations available but the clinical symptoms and the very fact that the fever responded well to the antimalarial treatment, proved malaria quite easily. From the pyrimethamine group 182 persons took regularly one tablet of Tindurin obtained from Chemical Works of Gedeon Richter containing 25 mg of pyrimethamine, out of which 3 had parasitologically positive malaria infection and in further 21 cases malaria was probable.

Table I. Effectivity of chemoprophylaxis

Drug	Number of persons	malaria		Percentage
		verified	non-verified	
Chloroquine	146	2	11	8.9
Pyrimethamine	182	2	21	13.2
Combinations	94	-	11	11.7
Proguanil	19	-	3	15.7
Irregularly	206	15	22	17.9
No drug taken	53	6	13	35.8

In the drug-combination taking group patients took one tablet of Fansidar /obtained from Hoffmann La Roche/ or Maloprim /obtained from Wellcome/ or Bayrena and Tindurin /Chemical Works of Gedeon Richter/ weekly. Among the 94 persons 11 probable malaria cases were found but no parasitologically

positive ones. Among the 19 subjects taking Proguanil in a daily dose of 100 mg 3 probable malaria cases were found but no parasitologically positive ones.

Out of the investigated 206 subjects in the group taking chemoprophylactics irregularly we found 15 parasitologically verified and 22 probable malaria cases.

In spite of medical advice given to the group in question 53 subjects took no chemoprophylactics whatsoever. 6 cases were parasitologically verified and 13 raised the suspicion of malaria. Table II shows the results of the P.falciparum IFAT.

Table II. Results of P. falciparum IFAT

Drug	Number of persons	IFAT positive	
		number	percentage
Chloroquine	146	2	1.4
Pyrimethamine	182	1	0.55
Combinations	94	2	2.1
Proguanil	19	-	-
Irregularly	206	14	6.8
No drug taken	53	4	7.5

2 P.falc. IgG positive cases were found in the chloroquine taking group, 1 in the pyrimethamine taking group, 2 in the combinations taking one, no positive cases were found in the proguanil taking group, 14 in the group taking the drugs irregularly, and 4 in the no-drug-taking one, out of which 5 were IgM positive, too. The rate of positive cases are 1.4, 0.55, 2.1, 0.0, 6.8, and 7.5 respectively.

Table III shows the geographic scattering of malaria distribution according to which the highest rate of malaria infection occurred in Black Africa most of all in West Africa and also in the Far East.

Table III. Distribution of the exposure to malaria

Region	Number of persons	IFAT positive	
		number	percentage
East Africa and Central Africa	149	5	3.4
Western Africa	239	lo	4.2
North Africa and the Middle East	177	2	1.1
The Far East	64	6	9.3
South America	38	-	-
Central America	lo	-	-

Up to the present day no ideal chemoprophylactics are available that would give a loo% protection against malaria. Contrary to the above, those who took chemoprophylactics regularly had malaria infections in a far lower rate. We would like to emphasize that in the group taking antifolic combinations there were no parasitologically verified malaria although the IFAT verified P. falciparum infections in two cases. In view of severe side-effects /neutropenia/ chemoprophylactics should not be administered for a long time (5,6). Similarly, prophylactics taken irregularly do not provide the necessary protection.

It is well known that the antibodies against P. vivax either do not or give only a weak cross-reaction with P. falciparum antigens. We still find the number of cases treated with malaria too high. The facts were considered in the same way in malaria-endemic areas where parasitological diagnosis can be hardly set up. There were only 26 parasitologically verified malaria cases out of which in 8 cases we set up the parasitological diagnosis because the patients were admitted to our Clinic with high fever after arriving home. Three of them proved to be P. falciparum infection the rest were P. vivax.

We assume that by using P. falciparum IFAT Spot Test a previous P. falciparum infection can be proved and the anamnestic data can be made more objective. On the basis of our examinations the rate of the parasitologically verified and probable malaria cases and those of IFAT positive ones are in good correlation in each group investigated.

Our presented results also support the fact that it is not the type of chemoprophylactic that matters in the first place but rather its regular administration.

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PROTOZOAN INFECTIONS IN THE GDR. RESULTS AND COMMENTS

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The increasing tendency with regard to parasite imports from the tropics and subtropics, furthermore protozoonoses widely distributed as urogenital trichomoniasis, especially intestinal protozoan infections, for instance giardiasis, and finally toxoplasmosis, posed some important problems for parasitological institutions in the GDR. Since 1975 the occurrence of different protozoan infections among indigenous populations and foreigners coming from outside was systematically supervised and documented in the GDR Reference Laboratory for Malaria and other Protozoan Diseases.

The techniques mainly used in our protozoological laboratories were the following:

- stained stool smear,
- thin and thick blood film,
- native preparations of vaginal and urethral smears,
- immunofluorescence reaction for serological diagnostics of toxoplasmosis.

During a period of about 10 years the frequencies of giardiasis ranged on an average from about 1.5% to 3.0%. Under special circumstances, in children's homes or among people who had contacts with regions of the warm climate, higher incidences up to about 12% were registered.

Entamoeba histolytica was found on an average in about 1.8%, the maximum frequencies, however, increased up to 19%. This phenomenon for instance was observed in children communities, mainly in those of psychiatric institutions. Furthermore, especially this

parasite could more frequently be detected among persons who spent some time in southern regions.

The percentages determined for Dientamoeba fragilis increased up to 27.0%. Such high numbers have been found only in a few labs using the cultivation technique, whereas in the majority only low incidences were reported, as it can be seen in the average frequencies determined (1-5%).

As to the clinical state of intestinal protozoan infections, there is no doubt about the facultative pathogenicity of Giardia lamblia. Beside this species in some cases also Dientamoeba fragilis and Entamoeba coli were supposed to produce pathogenic influences. Entamoeba histolytica presented pathogenic properties only in such patients who acquired the infection in regions of the warm climate.

The findings of Trichomonas vaginalis, which is widely distributed among the sexually active ages increased in number during the period from 1975 to 1980, as it was demonstrated by results of vaginal smears taken from patients of gynaecological and dermatovenereological clinics and practices. The maximum frequency, more than 30% in 1980, underlined the great importance of this venereal infectious disease. Later on the frequencies decreased significantly. This interesting phenomenon could be a result of the higher attention given to this infection in recent years and of the more intensive prophylaxis in this field.

The numbers of toxoplasma antibody carriers presenting high titres of the immunofluorescence test or showing seroconversion, range from about 1% up to 3.5%. In about half of the patients having an acute toxoplasmosis can it be supposed.

We are going to arrange a programme to prevent Toxoplasma primoinfections during pregnancy as it was done previously in other countries.

Up to 1982 the number of malaria imports in the GDR increased continuously (more than 80 cases in 1982), whereas later on a decreasing tendency was observed. Especially in this field extensive activities are carried out in the direction of a higher quality of diagnostics and therapy.

Regular training courses for the laboratory staff are organized including instructions on the in vivo test for detecting drug resistant malaria infections.

Within the frames of special research programmes investigations were started to detect Limax amoebae infections in the nasopharynx of children who had contacts with highly polluted water during swimming and watersports. In about 5-6%, Acanthamoeba could be found but related symptoms occurred only rarely.

Furthermore, among old people (n = 40) suffering from chronic diseases of the respiratory tract in 3 patients similar infections could be proved. After a specific antibiotic therapy the symptoms disappeared and the protozoological state was found to be negative. Later on, in 5 other cases Acanthamoeba species were isolated. It cannot be excluded that those amoeba, probably in interaction with a specific adapted bacterial flora, have pathogenic influences in chronic diseases on different organs. Therefore in the future more attention should be devoted to the occurrence of Acanthamoeba infections especially in such illnesses.

Concluding from all results and experiences it is necessary to include specific lines of protozoology into medical programmes more extensively than it was done in the past. As to this, it seems to be urgent to improve the diagnostic capacity of various medical institutions and to intensify the antiparasitic action programmes.

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ACTIVITY OF SOME AMIDINE TYPES AGAINST TRYPANOSOMATID FLAGELLATES IN VITRO

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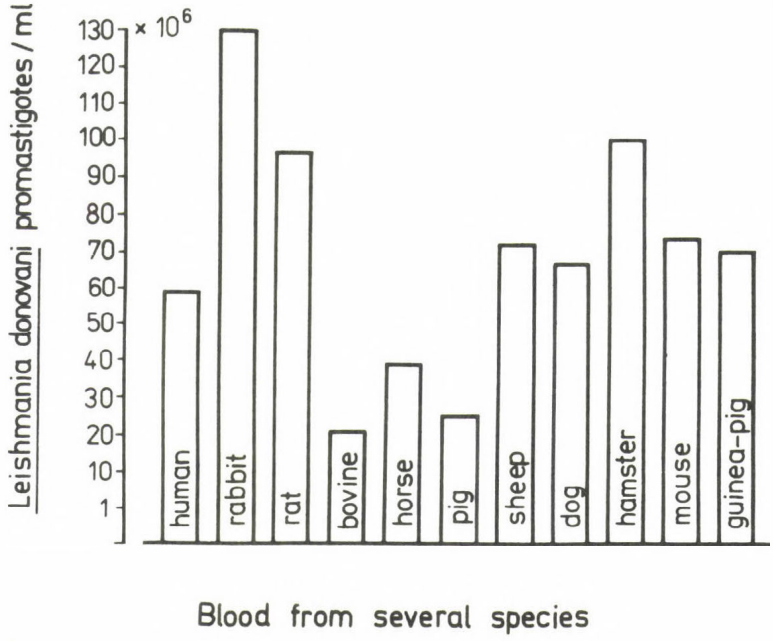
Progress in the development of new drugs for the treatment of trypanosomiasis and leishmaniasis has been slow. No effective new drugs for the treatment have been introduced into the field for over 25 years. The problems in chemoprophylaxis and chemotherapy of Trypanosoma and Leishmania infections relate to the toxicity of available drugs and drug resistance. There is a clear need for the development of new classes of potential drugs (1).

In a primary screen 60 amidines and congeners were tested for their in vitro action on the mobility, morphology and survival of promastigotes of Leishmania donovani, epimastigotes of Trypanosoma cruzi and trypomastigotes of Trypanosoma gambiense.

The effect of test substances on pro-, epi- and trypomastigotes was assessed by incubating organisms at 22°C with serial dilutions of drugs in test tubes. The range of concentrations used routinely in the assay was 0,1 to 10 microgrammes/ml. At various intervals (3, 12 and 24 hours) small aliquots were withdrawn for microscopic examination. In control tubes all of the organisms were active and morphologically normal in their media.

Leishmania donovani and Trypanosoma cruzi were cultured on NNN medium base with HEPES-buffered overlay, consisting of 3 tissue culture media (RPMI, Parker and Leibovitz L 15), supplemented with 2 per cent blood. Blood from several species, including human, rabbit, rat, bovine, horse and

others were evaluated for their ability to support optimal growth of the parasites in vitro. Rabbit blood was most effective (see table).



Only cultures in log phase, showing approximately 100 % increase in population in 24 hours were used. Trypanosoma gambiense trypomastigotes were taken from the first raising parasitaemia of infected mice. The parasites were separated from red blood cells by centrifugation and suspended in Ringer-glucose solution as a test medium. The number of parasites was adjusted to contain 5 millions/ml. They were examined microscopically to assess their mobility. In the course of testing 60 compounds for antiparasitic activity, 19 positive results were obtained with a number of chemical classes which are of interest. They include mono-

bis-benzylidencycloalkanone and naphthamidine compounds. There is considerable interest in the further development of compounds within these classes as potential new drugs. Some compounds showed curative activity only at toxic levels and were not worthy of further extensive testing. Several disadvantages are remarkable for in vitro models using promastigotes and epimastigotes in axenic culture, but this stage in the life cycle is not susceptible to some drugs.

For example, the pentavalent arsenical, tryparsamide, was inactive in vitro. This finding is consistent with the hypothesis that pentavalent arsenicals must first be reduced to the trivalent state in vivo to exert their antitrypanosomal activity. Suramin, sodium stibogluconate and meglumine antimoniate were also inactive in vitro.

WILLIAMSON (2) found in a similar in vitro system that suramin had less effect on the motility of the trypanosome than on infectivity.

Endocytosis is needed for the drug to be bound to plasma proteins before it can exert its antitrypanosomal activity. The 3-hour incubation period in the present system may not allow sufficient time for the drug to enter the parasite and exert its biological action. Alternatively, the drug may require in vivo metabolism to an active form and therefore its activity would not be detected by a simple in vitro test system.

However, the quantitative nature of the in vitro assay may allow more precise selection of the active compounds for further investigations (3). When employed as described, the in vitro test is capable of detecting antiparasitic actions for many compounds with different modes of action. Its potential for screening large numbers of compounds and for investigating the comparative activity of chemical analogues, a number of other uses of the in vitro system are under consideration. The quantitative evaluation of synergistic activity for various combinations of antiparasitic agents may be more readily achievable in vitro than by standard in vivo

methods. It is also of considerable value to be able to measure exactly the level of activity in human plasma specimens obtained following the administration of a potential new drug in clinical trials. For these reasons, the in vitro antiprotozoal test system may be an integral part of the search for new drugs.

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CLOSING ADDRESS

Ladies and Gentlemen,

Our memorial session and scientific meeting for the centenary of the birth of József Gelei has now come to an end. I have the honour of evaluating the work done here. Our conference had 120 participants from Hungary and abroad. Eighty-three lectures were delivered.

It was particularly rewarding for the Board of this Conference of Protozoology that guests from 19 countries were present at the meeting. In all three sections the lectures were of high level, of international standing. The congenial atmosphere of the discussions, the constructive and critical contributions by the participants no doubt were a stimulus for further research.

I take this opportunity to announce to Prof. J.J. Paulin, Secretary of the Society of Protozoologists, our intention to join the Society. I am sure with our membership and our access to the Journal of Protozoology our research and publicity for our work will be promoted.

On behalf of the Biological Section of the Hungarian Academy of Sciences and the Section of Protozoology of the Hungarian Biological Society I should like to thank you very much for your participation. I hope you have enjoyed our hospitality and that you will visit us in the future, too.

I declare the conference closed.

Magdolna Cs. Bereczky
President of the
Organizing Committee

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